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CHEMICAL SYNTHESIS OF SUCROSE ANALOGUES AND THE STUDY OF
THEIR INHIBITION OF DEXTRANSUCRASE

Iowa State University

PH.D.

1980

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Chemical synthesis of sucrose analogues and the
study of their inhibition of dextransucrase

by

John Nicholas Zikopoulos

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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DEDICATION

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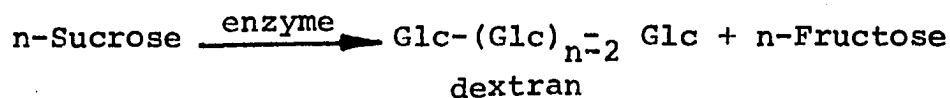
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I. INTRODUCTION

Recently there has been a surge in interest about sugars, especially sucrose and its connection with diet and dental caries. This is by no means a new interest as witnessed by the fact that Aristotle (384-322 B.C.) wondered about the connection between the deteriorating teeth of Athenian children and their diet containing large amounts of soft and sweet figs (1). What is new, besides candy bars, is that now the biochemical mechanisms involved in these processes are being elucidated and will thereby provide the foundations for the establishment of a means of control.

This introduction will focus mainly on the specifics of a model enzymatic system, namely dextransucrase (E.C.2.4.5) an extracellular enzyme produced, among others, by the bacterium Leuconostoc mesenteroides B512-F, and the chemistry of sucrose and other analogous carbohydrates and their derivatives. Dextransucrase, as was stated earlier, is an extracellular enzyme elaborated by bacteria in the Lactobacillaceae group. The enzyme catalyzes the reaction shown in Figure 1.

Dextran is a glucan with main chain linkages consisting of $\alpha 1 \rightarrow 6$ bonds and branch points of $\alpha 1 \rightarrow 2$, $\alpha 1 \rightarrow 3$, or $\alpha 1 \rightarrow 4$ depending upon the source of the particular enzyme



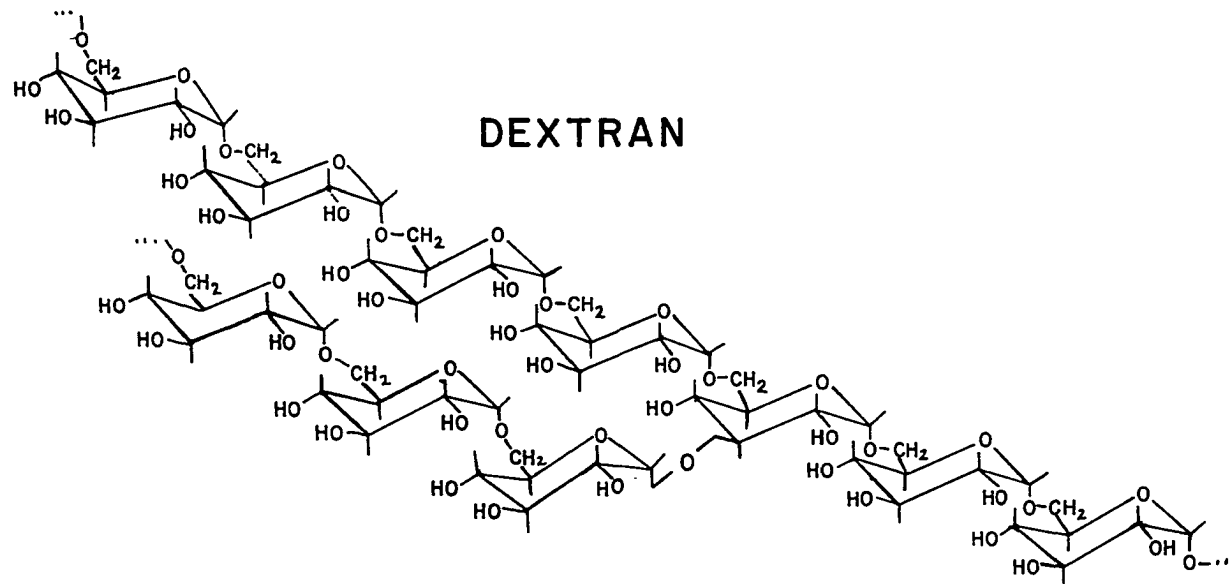
Glc \equiv Glucose

enzyme \equiv dextransucrase

Figure 1. Reaction catalyzed by dextransucrase.

catalyzing the reaction. The enzyme from Leuconostoc mesenteroides B512-F produces a dextran which consists of 95% $\alpha 1 \rightarrow 6$ linkages and 5% $\alpha 1 \rightarrow 3$ branch linkages. The structure is illustrated in Figure 2. This dextran has been known for many years and has found some use as a blood plasma extender and as the source polysaccharide used in the production of the various molecular sieves known as Sephadex. A renewed interest in dextran has developed due largely to the fact that it has been identified as a major constituent of dental plaque where it is responsible for the adhesion properties of the plaque which bonds bacteria, food particles, and maintains an anaerobic and acidic microenvironment on the surface of the human tooth (2). These conditions allow the bacteria to carry out anaerobic fermentation which results in the excretion of various organic acids by the organisms as metabolic end products. The acidic environment is maintained by the plaque which does not allow the washing away or diffusion of the acid from the surface of

Figure 2. The partial structure of Leuconostoc
mesenteroides B512-F dextran.



Leuconostoc mesenteroides

B-512 Dextran

95% α -1 \rightarrow 6 linkages

5% α -1 \rightarrow 3 branch linkages

the tooth. The organic acids then act upon the hydroxyl apatite matrix of the tooth inducing solubilization and consequent demineralization. This is the beginning of a cavity, a phenomenon experienced by almost all of present day mankind.

A variety of bacteria inhabit the mouth, but not all are cariogenic. It appears that in order to be cariogenic the bacterium must be capable of producing dextransucrase and consequently synthesizing dextran. Dextran is not the only polysaccharide found in plaque; however, it does appear to be of major importance. Mutan, a polysaccharide, consisting of an $\alpha 1 \rightarrow 3$ linked glucopyranosyl main chain with $\alpha 1 \rightarrow 6$ branch points is found in plaque along with levan a $\beta 2 \rightarrow 6$ linked fructofuranosyl polysaccharide, which may serve the function of a reserve source of carbohydrate food for the bacteria (3). In addition to these polysaccharides a glycogen-like polysaccharide is produced from sucrose by a bacterium inhabiting the human throat known as Niserea perflava (4). Each of the polysaccharides referred to, i.e., dextran, mutan, levan, and the glycogen-like polymer is synthesized by a specific enzyme utilizing the common substrate, namely sucrose. All of these polysaccharides and their corresponding enzymes are extracellular except for the glycogen and the corresponding amylosucrase enzyme. The reactions catalyzed by each of these enzymes is illustrated in Figure 3.

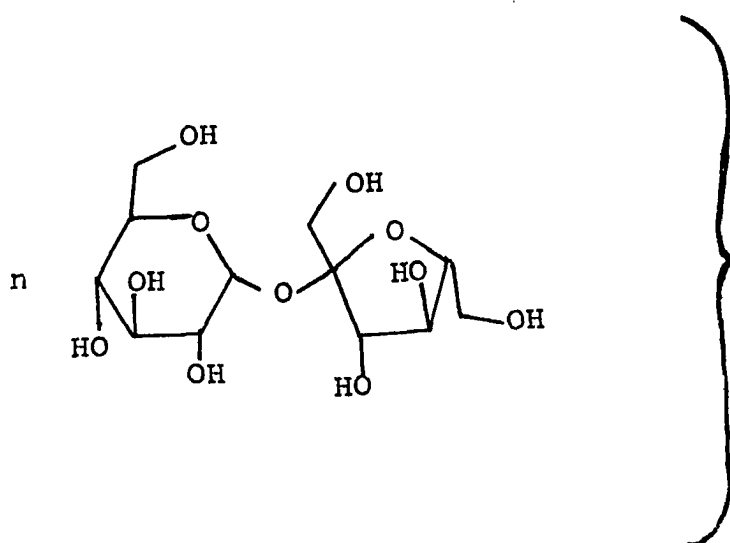
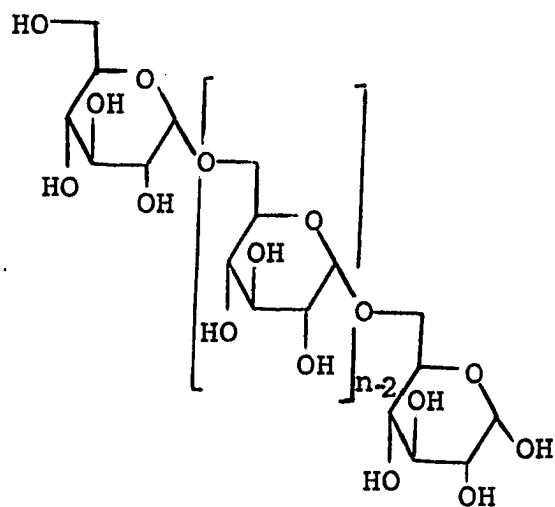


Figure 3. Polymerization reactions catalyzed by destransucrase, sucrase, levansucrase, and amylosucrase. The products shown do not indicate branching.

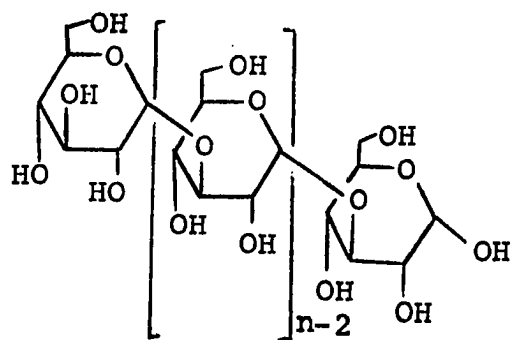
Dextranucrase

$n[\text{Fructose}]$



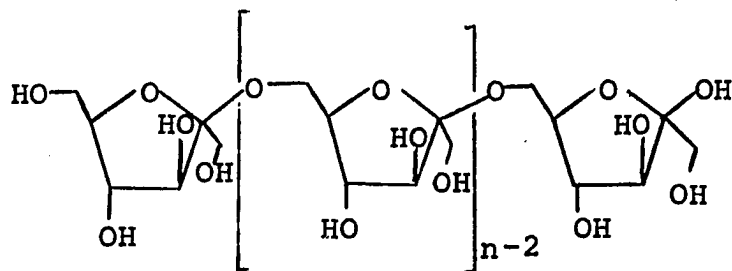
Mutansucrase

$n[\text{Fructose}]$



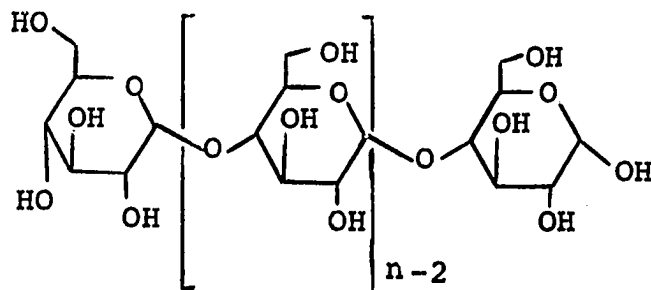
Levansucrase

$n[\text{Glucose}]$



Amylosucrase

$n[\text{Fructose}]$



Examination of the substrates and reaction products yields some information about the corresponding enzyme; however, a more detailed analysis is required to shed light on the mechanism of action of any of these enzymes. Such studies have been and are presently underway.

I would like now to focus on a more detailed account of such studies for the enzyme dextranucrase especially the work of Dr. John F. Robyt and coworkers.

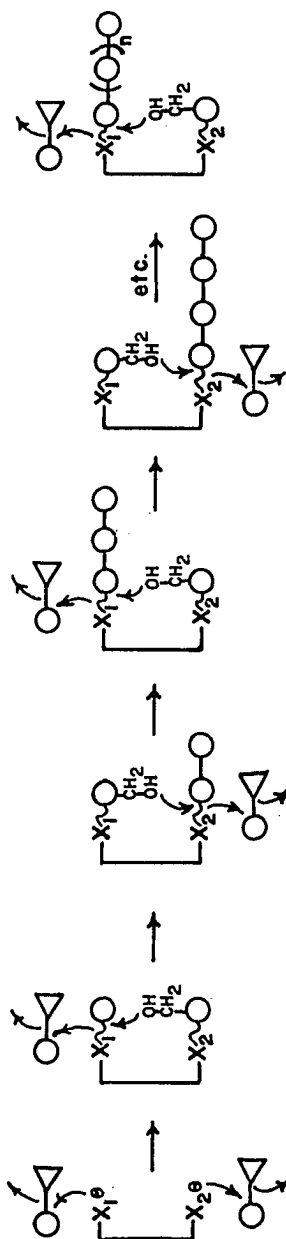
Robyt, Kimble and Walseth (5) in a study of the direction of dextran synthesis by dextranucrase from Leuconostoc mesenteroides B512-F demonstrated that synthesis occurs from the reducing end by an insertion mechanism. These conclusions were arrived at by examining the enzymatic reaction utilizing a modified pulse-chase technique with radioactively labelled ^{14}C -sucrose as the substrate. This work demonstrated that cell-bound dextranucrase and Bio-Gel-coupled-dextranucrase could be labelled by treatment with ^{14}C -sucrose and that this label could be removed by treatment of these two systems with buffer at pH = 2.0, followed by heating to 95°C for ten minutes. Two types of radioactive products could be isolated under these conditions, namely dextran and glucose. Treatment of dextranucrase with ^{14}C -sucrose (a pulse) followed by incubation with nonradioactive sucrose (a chase) and the isolation of the polysaccharide which was reduced with sodium

borohydride and acid hydrolysis showed that in a pulse the ratio of ^{14}C -sorbitol to ^{14}C -glucose was 1 to 1, and in a pulse followed by a chase the ratio was 1 to 100. These data were the basis on which it was concluded that the dextran was biosynthesized by the transfer of glucosyl units from sucrose to the reducing end of the growing dextran chain. A mechanism was proposed to explain these results and it is shown in Figure 4.

The mechanism was explained in the Robyt et al. (5) paper by the following statement:

"It is proposed that dextransucrase forms an enzymatically active covalent complex with glucose and dextran and that the glucose is inserted between the enzyme and the dextran by a nucleophilic attack of the $\text{C}_6\text{-OH}$ of glucose onto C_1 of the dextran forming an $\alpha 1+6$ glucosidic bond. This releases one of the nucleophilic groups at the active site which attacks sucrose to give an enzyme-glucosyl complex with concomitant release of fructose into the medium. The $\text{C}_6\text{-OH}$ of this glucose then repeats the process of attacking C_1 of dextran. Dextran is built up by extrusion from the enzyme when glucose units are transferred from sucrose to the active site and inserted between the enzyme and the reducing end of the dextran polymer."

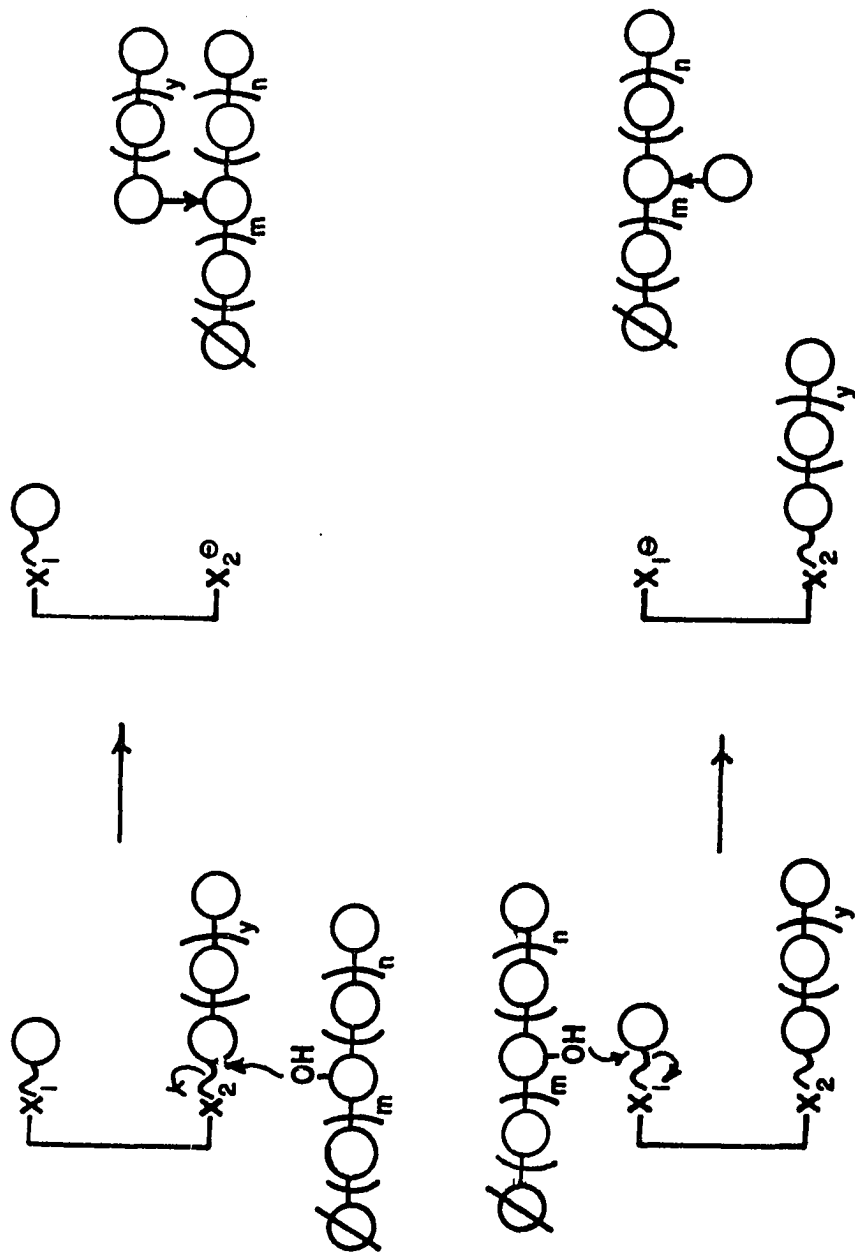
Figure 4. Mechanism for dextran biosynthesis by dextran sucrose proposed by Robyt et al. (5) X_1 and X_2 represent nucleophilic groups at the active site of the enzyme. \bigcirc represents glucose, \triangleleft represents fructose, $\bigcirc\triangleleft$ is sucrose, and $-\bigcirc-\bigcirc-$ is two glucose units linked by an $\alpha 1 \rightarrow 6$ glucosidic bond.



Further work by Robyt and Taniguchi (6) established the mode of biosynthesis of the branch linkages in the dextran molecule by the action of dextransucrase. In this work, Bio-Gel-coupled-dextransucrase was utilized to produce so called charged enzyme, which had ^{14}C -labelled dextran. This was accomplished by incubating the immobilized enzyme with ^{14}C -sucrose in buffer and subsequently washing the resulting complex with buffer to yield an enzyme ^{14}C -dextranosyl complex. This complex (postulated to be covalent in nature) was incubated with a low molecular weight non-labelled dextran. This treatment resulted in the complete release of all the label from the immobilized enzyme with the production of branched and labelled dextran.

Subsequent acetolysis and work-up resulted in the formation of radioactive nigerose, an α -D-glucopyranosyl- α 1 \rightarrow 3-glucopyranoside. Reduction of this compound with sodium borohydride followed by acid hydrolysis resulted in the release of the radioactivity in the form of ^{14}C -glucose, indicating that the label was exclusively in the nonreducing end of the nigerose. This result shows that the ^{14}C -dextran was released from the enzyme by the low molecular weight non-labelled dextran with the formation of a new α 1 \rightarrow 3 linkage. The mechanism which has been proposed and is consistent with these facts and the earlier work of Robyt, Kimble and Walseth is shown in Figure 5.

Figure 5. Acceptor reactions of dextransucrase giving rise to branching in the dextran molecule. From Robyt and Taniguchi (6).



The mechanism for forming $\alpha 1 \rightarrow 3$ branch linkages was described by Robyt and Taniguchi (6) by the following statement:

"A mechanism is proposed in which a C_3 -OH on an acceptor dextran acts as a nucleophile on C_1 of the reducing end of a dextranosyl-dextranase complex, thereby displacing dextran from dextranase and forming an $\alpha 1 \rightarrow 3$ branch linkage. It is argued that the biosynthesis of the branched linkages does not require a separate branching enzyme but can take place by reaction of an acceptor dextran with a dextranosyl-dextranase complex."

Earlier work by Koepsell and Tsuchiya (7) and Tsuchiya et al. (8), had shown that low molecular weight carbohydrates when added to a reaction mixture of dextranase and sucrose resulted in the formation of a lower molecular weight dextran than is observed without the addition of the low molecular weight sugars. In addition to this, a series of oligosaccharides were produced in large amounts which were not ordinarily seen when the only added carbohydrate was sucrose.

These observations were explained as a result of primer requirements of dextranase in a manner similar to that observed with the enzyme glycogen phosphorylase. It seems that the reason for this hypothesis was that it was generally believed that the substrate, i.e., sucrose had to be present

for the reactions with the low molecular weight carbohydrates to proceed.

Robyt and Walseth (9) showed that the acceptor reactions could occur both in the presence and in the absence of sucrose. The low molecular weight acceptors chosen for this study were D- $[^{14}\text{C}]$ -glucose, D- $[^{14}\text{C}]$ -fructose and ^{14}C -reducing-end-labelled maltose. Purified dextransucrase was incubated with sucrose and then the unreacted sucrose and newly produced fructose were removed from the enzyme via column chromatography on Bio-Gel P-6. This resulted in the formation of charged enzyme, i.e., dextransucrase containing covalently linked dextran and covalently linked glucose. The charged enzyme was reacted with each of the three acceptors both in the presence and in the absence of sucrose.

In the absence of sucrose, a labelled dextran was obtained along with a labelled oligosaccharide. In each of the three cases the acceptor was incorporated into the dextran and the oligosaccharide at the reducing end.

Similar results were obtained in the presence of sucrose except that higher yields of the oligosaccharides were obtained. In the case of glucose and of maltose, a homologous series of oligosaccharides was obtained. The proposal was made that the acceptor reactions proceed by a nucleophilic attack of a specific hydroxyl group of the acceptor on the enzyme-glucosyl and enzyme-dextransosyl

covalent complexes. Robyt and Walseth concluded that the acceptor reactions serve to terminate polymerization and that acceptors do not act as primers (9). The question of primer requirements was brought forth again in the case of dextransucrase from Streptococcus mutans OMZ 176 (10). The observation had been made by some investigators that the addition of dextran to dextransucrase from Streptococcus mutans produced an apparent stimulation of activity. Some investigators in this area felt that the stimulation in activity was evidence for the requirement of primer by this enzyme (11).

In the case of dextransucrase from Leuconostoc mesenteroides one could easily explain these activation effects by the acceptor reactions resulting in the formation of branched linkages.

Germaine et al. (11) deduced from their experiments that dextransucrase from Streptococcus mutans OMZ 176 required a primer with reaction occurring at the nonreducing end of the primer.

Robyt and Corrigan, through specific modifications of dextran "primers" at the nonreducing end, showed that a free nonreducing end was not necessary to produce the observed activation effects (10). These effects could have an alternative explanation such as allosteric effects or acceptor reactions or a combination of both of these phenomena. The

modifications involved chemical and or enzymatic treatments of the dextran to be tested as a primer. In one case, low molecular weight dextran was treated with 2,4,6-triisopropylbenzenesulfonyl chloride which is sterically favored for reaction with primary hydroxyl groups on a glucosyl residue as compared to the secondary hydroxyl groups on a glucosyl residue.

This derivatization did not significantly alter the activation effects of the low molecular weight dextran on dextransucrase from Streptococcus mutans OMZ 176. Dextran treated with exodextranase, which produced very short outer chains, also gave the same results. Dextran, which had first been treated with exodextranase followed by reaction with 2,4,6-triisopropylbenzenesulfonyl chloride, also gave similar activation results with dextransucrase.

Due to these facts, a primer function cannot be attributed to the added dextran, however, an allosteric and/or acceptor reaction still fits the observable data. It appears from this work that there is no requirement for primer in the synthesis of dextran by dextransucrase and that the mechanism of chain formation can be de novo from nascent enzyme, i.e., enzyme containing no glucose or dextran (10).

Further work on dextransucrase from Leuconostoc mesenteroides B512-F by Robyt and Walseth (12) has shown that this enzyme is a metallo-enzyme requiring calcium ions for

activity. The enzyme displays maximum activity with the addition of 0.005% w/v of calcium chloride. Inhibition of dextransucrase activity could be observed when the enzyme was treated with a chelating agent such as EDTA or when another ion such as Zn^{+2} was added instead of Ca^{+2} (12). Studies involved with the inhibition of dextransucrase have been somewhat limited. Figures and Edwards (13) carried out a study utilizing α -D-glucopyranosyl fluoride as a substrate for dextransucrase from Streptococcus mutans in which they tested a variety of carbohydrate derivatives both as substrates and inhibitors. The most potent inhibitor of the 29 reported compounds tested was 3-deoxy-D-ribohexose (3-deoxy-D-glucose) which showed 43% inhibition at 25 mM and 50 mM concentrations.

A claim was made that 6,6'-dideoxysucrose did not inhibit the enzyme; however, it did protect it from acidic denaturation. No details were described. Another notable lack of inhibition was observed by the authors with 6-deoxy- α -D-glucopyranosyl fluoride. It is interesting to note that dextransucrase showed substrate inhibition using α -D-glucopyranosyl fluoride at a concentration greater than 30 mM. A similar observation was made by Genghof and Hehre (14) on dextransucrase from Leuconostoc mesenteroides B512-F.

Dextransucrase from Leuconostoc mesenteroides B512-F also displays substrate inhibition with sucrose at concen-

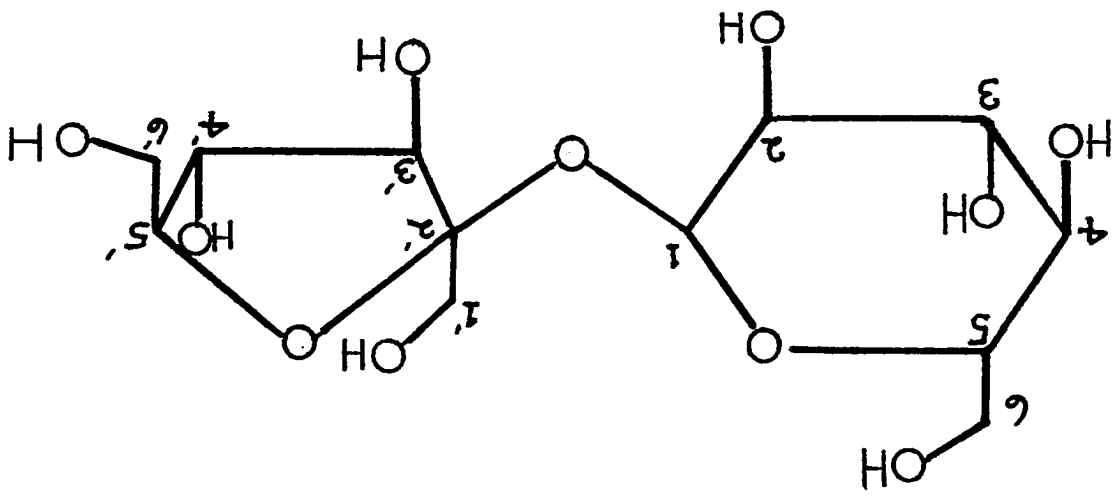
trations greater than 200 mM according to Genghof and Hehre (14). Other than the inhibitor studies by Figures and Edwards (13) and those resulting from EDTA and/or zinc ions (and, of course, such general enzyme inhibitors as mercury ions, lead, and cadmium ions) Robyt and Walseth (12), inhibition studies with substrate analogues have not been made. An initial consideration of substrate analogues is their availability.

The chemistry of sucrose (sucrochemistry) has been studied for several years by several researchers; however, in the past decade there have been some advances made which have allowed for the synthesis of a variety of sucrose derivatives which were previously unknown.

A word about the sucrose molecule is in order at this point, prior to delving into the host of chemical changes that can be carried out on it. Sucrose or β -D-fructofuranosyl- α -D-glucopyranoside or α -D-glucopyranosyl- β -D-fructofuranoside has the structure shown in Figure 6. This substance is produced in ton quantities on a yearly basis and, in addition, is readily available in a high state of purity.

The chemistry of sucrose is complex with regard to the number of derivatives theoretically possible. Sucrose, however, portrays a certain degree of selectivity and the resulting derivatives, of course, have an even greater selectivity in the reactions they will undergo.

Figure 6. The structure of α -D-glucopyranosyl-
 β -D-fructofuranoside (sucrose).



There have been many derivatives of sucrose synthesized, especially in the past decade with the emergence of new and stereoselective reagents. Some of these derivatives will be discussed further; however, for more information the reader is directed to some excellent reviews on the subject (15-18).

Derivatization of sucrose on the primary hydroxyl groups is facilitated by their somewhat greater reactivity as compared to the secondary hydroxyl groups. As an example, it is possible to directly chlorinate positions 6 and 6' of sucrose by careful treatment with sulfuryl chloride in pyridine at low temperature (19).

There is, of course, reaction at the other positions; however, it is possible to obtain pure 6,6'-dideoxy-6,6'-dichlorosucrose by chromatography on a silica gel column. This selectivity has been enhanced further by use of sterically bulky reagents, such as triphenylchloromethane to yield the trityl ethers (20), triisopropylbenzenesulfonyl chloride to yield the tripsyl esters (21) and the combination of triphenylphosphine and a carbon tetrahalide to yield the corresponding deoxyhalogen derivative directly (22).

The halogen derivatives have been of great interest recently because of the fact that some are intensely sweet (23) and others have proven to affect sperm and have been shown as effective male birth control agents in rats (24).

Various chlorosucrose derivatives have been synthesized especially by Hough and coworkers (25). A common technique is the synthesis of the appropriate sulfonate ester of sucrose followed by esterification of the remaining hydroxyl groups. This derivative is then treated with an alkali metal chloride in a dipolar aprotic solvent such as dimethylformamide (DMF) or hexamethylphosphoramide (HMPA) to yield the chlorinated derivative which is subsequently deesterified to yield the free sucrose analogue.

An example of this is the synthesis of 6,1',6'-trideoxy-6,1',6'-trichlorosucrose as carried out by Hough et al. (25) and illustrated in Figure 7. Hough and Mufti (26) had earlier prepared 6,6'-dideoxy-6,6'-dichlorosucrose by the route shown in Figure 8.

Use of the appropriate method can yield mono, di, tri, or tetrasubstituted or higher substitutions of sucrose.

Some of the methods used in the synthesis of the chloro analogues of sucrose can be used in the synthesis of bromo and iodo analogues as well. As an example, the synthesis of 6,6'-dideoxy-6,6'-dibromosucrose has been achieved directly from sucrose by using triphenylphosphine and carbon tetrabromide in a manner analogous to the dichloro analogue synthesis as shown in Figure 9.

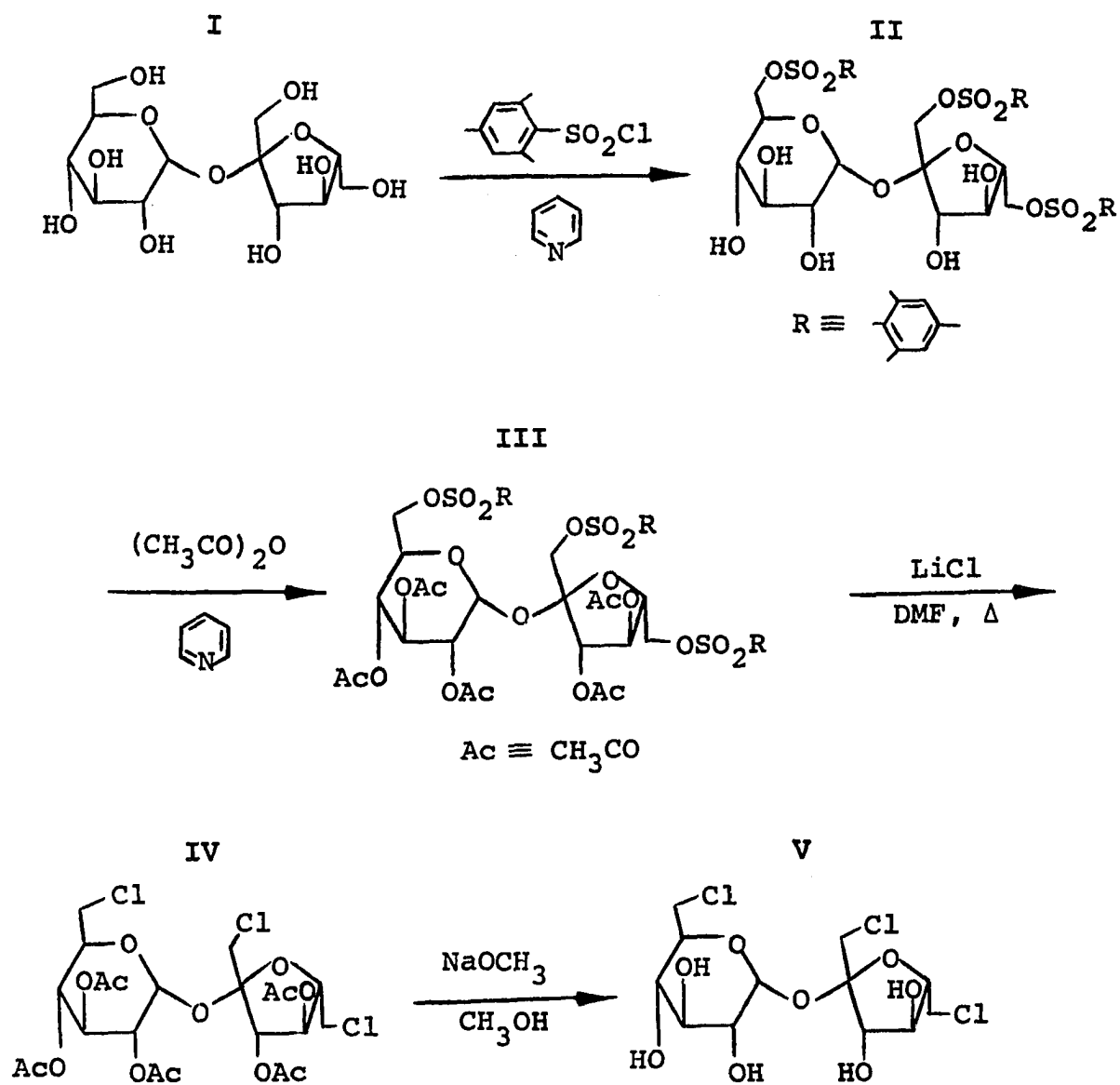
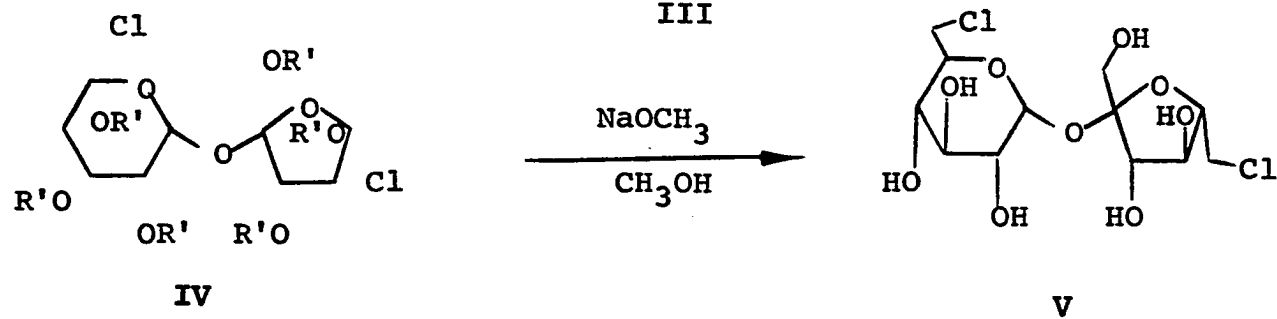
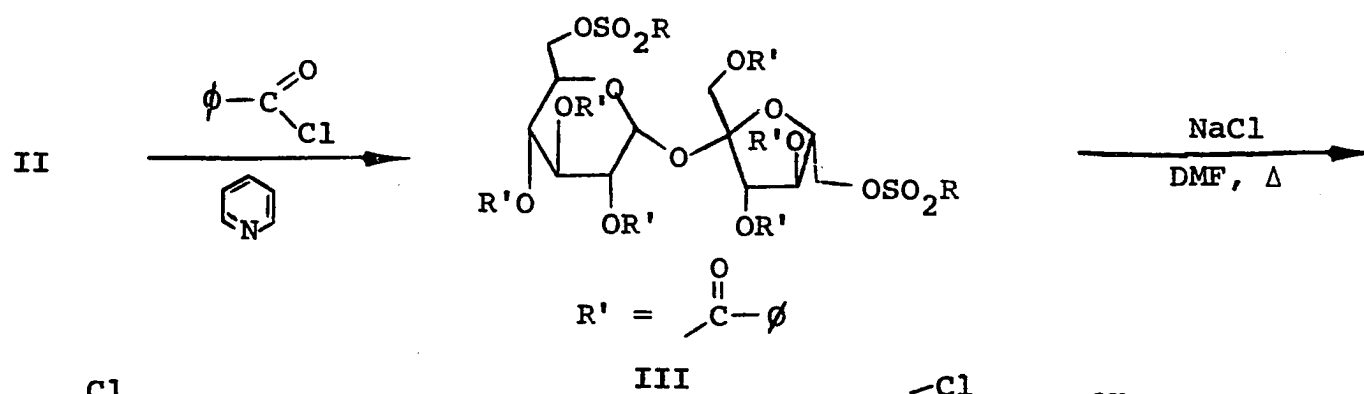
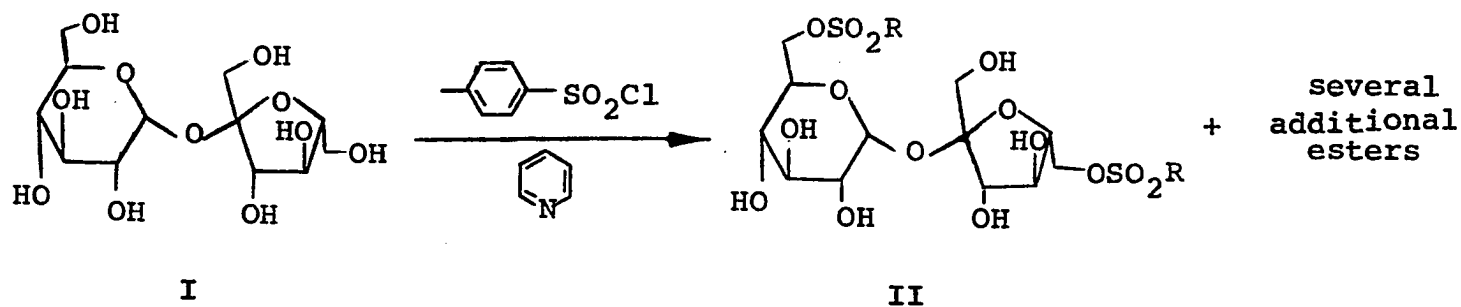


Figure 7. The synthesis of 6,1',6'-trideoxy-6,1',6'-trichlorosucrose as carried out by Hough et al. (25).

Figure 8. The synthesis of 6,6'-dichloro-
6,6'-dideoxysucrose as carried
out by Hough and Mufti (26).



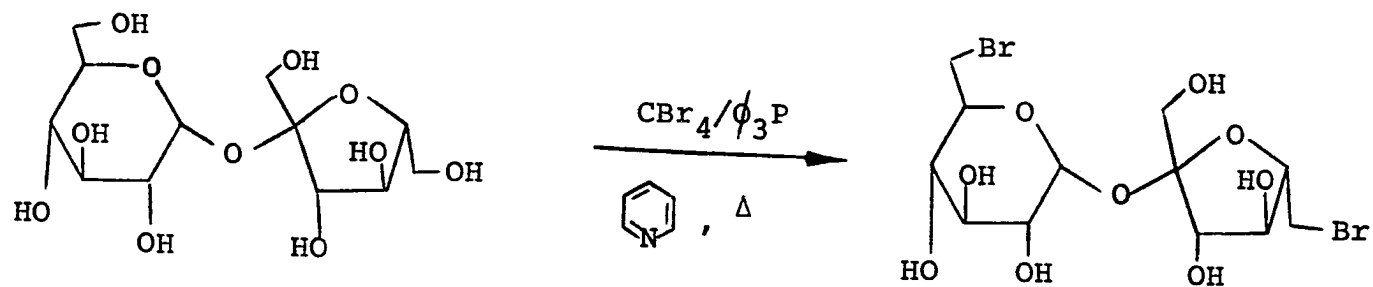


Figure 9. Synthesis of 6,6'-dideoxy-6,6'-dibromosucrose.

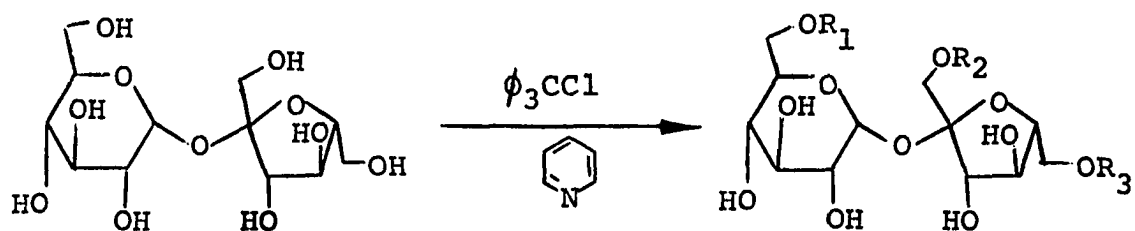
The same reaction would probably work for the synthesis of the diiodo analogue by using carbon tetraiodide instead of carbon tetrachloride or carbon tetrabromide.

Both the dibromo and diiodo sucrose analogues can be synthesized via the 6,6'-di-O-tosylate followed by nucleophilic displacement by alkali metal bromide or iodide. Similar techniques have been employed for the synthesis of azido, thio-cyanato, and deoxy analogues (27).

In addition to these di and tri substitutions on sucrose, it is possible to chromatographically select the proper mono-O-tosyl ester of sucrose in order to prepare mono substituted analogues of sucrose. An example of this is the preparation of 6-deoxy-6-iodo-2,3,4,1',3',4',6'-hepta-O-benzoylsucrose by treatment of the corresponding tosylate with sodium iodide in refluxing butanone (17).

In addition, Almquist and Reist (27, 28) have shown that with 6,1',6'-tri-O-triisopropylbenzenesulfonylsucrose as a starting material, it is possible to prepare mono and disubstituted sucrose derivatives.

One of the more useful reagents for the synthesis of mono, di, and trisubstituted sucrose derivatives has been triphenylchloromethane (tritylchloride). Reaction of sucrose with this sterically bulky reagent results in the formation of the products shown in Figure 10.



	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
I	$\text{C}_6\text{H}_5\text{C}-$	H-	H-
II	H-	$\text{C}_6\text{H}_5\text{C}-$	H-
III	H-	H-	$\text{C}_6\text{H}_5\text{C}-$
IV	$\text{C}_6\text{H}_5\text{C}-$	H-	$\text{C}_6\text{H}_5\text{C}-$
V	$\text{C}_6\text{H}_5\text{C}-$	$\text{C}_6\text{H}_5\text{C}-$	H-
VI	H-	$\text{C}_6\text{H}_5\text{C}-$	$\text{C}_6\text{H}_5\text{C}-$
VII	$\text{C}_6\text{H}_5\text{C}-$	$\text{C}_6\text{H}_5\text{C}-$	$\text{C}_6\text{H}_5\text{C}-$

Figure 10. List of products resulting from tritylation of sucrose in pyridine.

The ratios of the products vary with reaction conditions and it is possible to maximize the desired product or products by varying the ratio of trityl chloride to sucrose and varying the time of reaction.

The selectivity of the tritylation reaction and the relatively facile removal of the trityl groups from the sucrose molecule by mild acid conditions has led to extensive use of this reagent for the synthesis of novel derivatives of carbohydrates in general and sucrose in particular. Other reagents similar in nature to trityl chloride are available and have been used for similar purposes. Examples of these are methyl-t-butylsilyl chloride and diphenylmethylsilyl chloride.

Other methods that are available for the derivatization of specific points on the sucrose molecule are use of benzylidene derivatives and isopropylidene derivatives (29-32) which give 4,6 and 4',6' substitution.

II. EXPERIMENTAL

A. Materials

The following is a list of materials obtained from
J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Acetic anhydride

Sodium acetate

Xylose

Sodium methoxide

Raffinose

Mellibiose

Glucose

Fructose

Sucrose

Carbon tetrachloride

Pyridine

Triphenylchloromethane

Benzoyl chloride

Amberlite IR 120 (H⁺) ion exchange resin

Amberlite MB-3 ion exchange resin

Silica gel for column chromatography

Potassium fluoride

Lithium fluoride

Lithium chloride

p-toluenesulfonyl chloride

Dimethylformamide

Iodine

The following is a list of materials obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

Triphenylphosphine

Carbon tetrabromide

2,4,6-triisopropylbenzenesulfonyl chloride

Mesitylenesulfonyl chloride

Hexamethylphosphoramide

Diglyme

18-crown-6

Dibenzo-18-crown-6

Diglyme

The following were obtained from Amersham/Searle, Arlington Heights, Illinois.

2,5-diphenyloxazole, (Scintillation Grade) (PPO)

1,4-bis(2-(5-phenyloxazolyl))benzene, (Scintillation Grade) (POPOP)

^{14}C -[UL]-sucrose was obtained from New England Nuclear, Boston, Massachusetts.

Trifluoroacetic acid was obtained from Pierce Chemical Company, Rockford, Illinois.

32% Hydrogen bromide in glacial acetic acid was obtained from Eastman Kodak Company, Rochester, New York.

Aerobacter levanicum NRRL B-1678 was obtained from Northern Regional Research Laboratory, Peoria, Illinois.

B512-F dextranucrase was a gift from Dr. Timothy Walseth and Mr. Joe Couto from the laboratory of Dr. John F. Robyt, Iowa State University, Ames, Iowa.

Mutansucrase (Streptococcus mutans OMZ 176) was a gift from Dr. Alice Corrigan from the laboratory of Dr. John F. Robyt, Iowa State University, Ames, Iowa.

Diethylaminosulfur trifluoride was obtained from PCR, Inc., Gainesville, Florida.

Whatmann 3MM paper was obtained from Scientific Products Inc., Chicago, Illinois.

Thin layer chromatography plates were obtained from Analtech Inc., Newark, Delaware, and Eastman Kodak Company, Rochester, New York.

Fluorescent silica gel for column chromatography was obtained from ICN Nutritional Biochemicals Inc., Cleveland, Ohio.

All other materials were of reagent grade quality unless otherwise stated in the text.

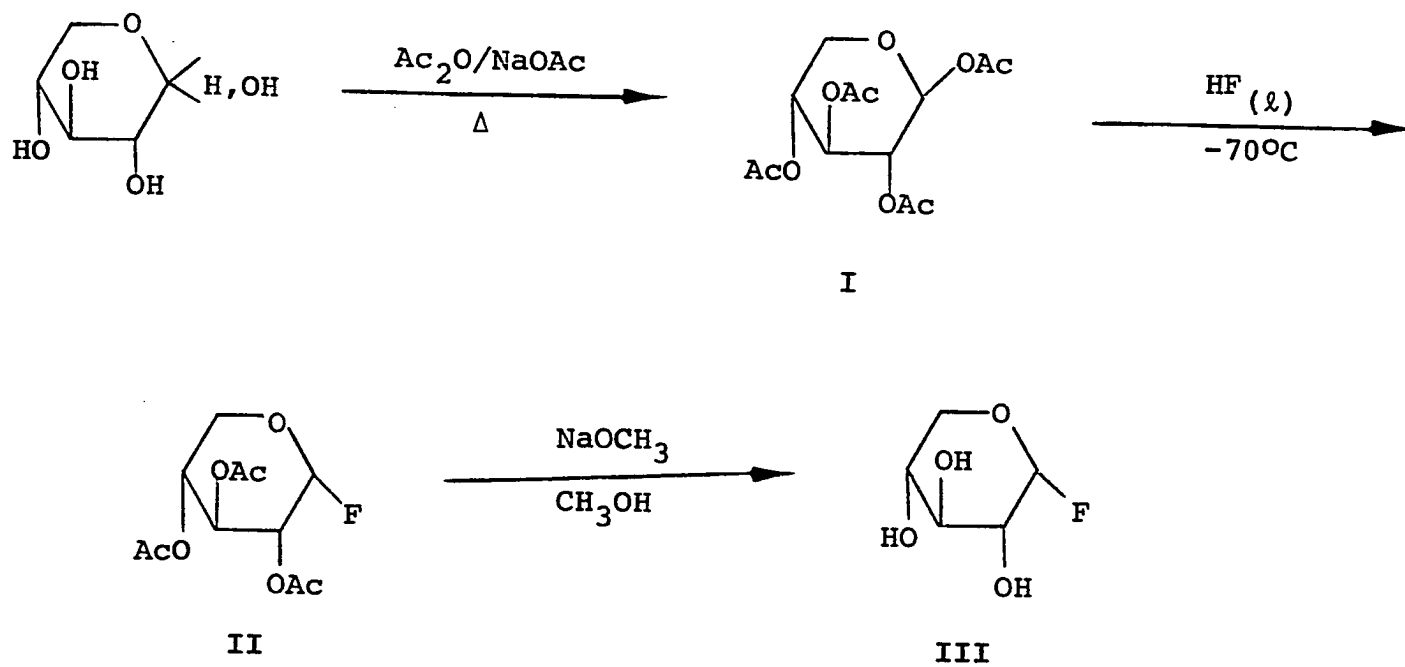
All melting points are uncorrected in this study.

B. Methods

1. Synthesis of α -D-xylopyranosyl fluoride

The preparation of the title compound was carried out in the manner described by Micheel et al. (33). An example of the preparation is given as follows (see Figure 11).

a. β -D-Xylopyranosyl tetracetate (I) Xylose (50 g)
was added in small portions to a mixture of acetic anhydride (350 ml) and sodium acetate (25 g) at a rate sufficient to maintain the exothermic reaction. Upon complete addition of the xylose to the reaction mixture, the final reaction mixture was heated to the boiling point and maintained at this temperature for approximately one minute. The mixture was allowed to cool to room temperature and then poured into ice water. A precipitate was formed and it was collected by filtration. The precipitate was dissolved in chloroform and the filtrate was extracted with chloroform. The chloroform solutions were combined and concentrated to form a thick syrup. This was dissolved in hot absolute ethanol, treated with decolorizing carbon and filtered. The filtrate upon cooling produced crystals which had a melting point range of 121-124°C. These were collected and recrystallized from hot absolute ethanol. The second batch of crystals had a melting point of 128-130°C. These were dried in a vacuum oven at 40°C overnight. The final yield was 32.5 g (30%) of β -D-xylopyranosyl tetraacetate (I).



$\text{Ac} = \text{CH}_3\text{CO}$

Figure 11. Synthesis of α -D-Xylopyranosyl fluoride.

b. α -D-Xylopyranosylfluoride triacetate (II) Compound I (10.0 g) was treated with 50 ml of anhydrous hydrogen fluoride for 15 to 20 minutes. The resulting reaction mixture was diluted with diethyl ether and the ether solution was washed with aqueous sodium bicarbonate and distilled water. The organic phase was removed, dried, and evaporated to a thick syrup.

c. α -D-Xylopyranosyl fluoride (III) Compound II as the syrup was dissolved in anhydrous methanol (50 ml) and treated with a 0.01 mM sodium methoxide solution in a conventional de-acetylation procedure. The reaction was quenched by adding solid CO₂ and the reaction mixture was evaporated to dryness. The resultant solid was extracted with hot ethylacetate, decolorized, filtered, and evaporated to dryness.

A ¹³C-NMR spectrum of xylose is shown in Figure 12 and a ¹³C-NMR spectrum of α -D-xylopyranosyl fluoride is shown in Figure 13.

2. Preparation of α -D-xylopyranosyl- β -D-fructofuranoside (Xylsucrose)

This procedure is derived from the work of Avigad et al. (34) who first prepared this substance enzymatically.

The xylsucrose was prepared as follows: Approximately one gram of raffinose and two grams of xylose were added to 10 ml of 20 mM pyridine acetate buffer (pH = 5.0); then to this mixture 0.2 g of packed, washed cells of Aerobacter

Figure 12. ^{13}C -NMR spectrum of D-Xylose
containing both α and β anomers.

PPM of Peaks

90.604
86.168
69.816
68.032
66.815
65.467
63.379
63.205
59.161
54.942
DMSO 32.808

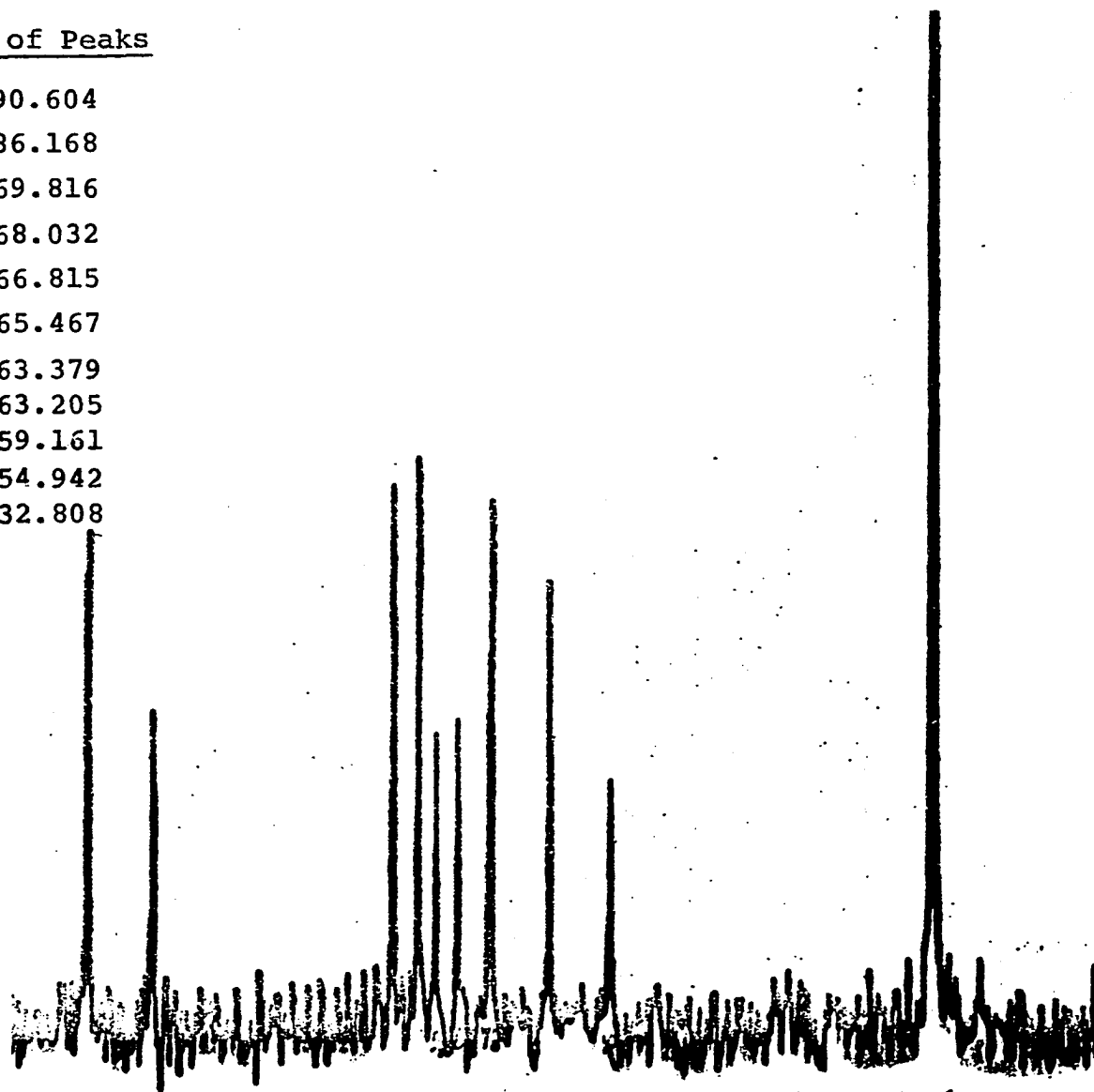


Figure 13. ^{13}C -NMR spectrum of α -D-xylopyranosyl fluoride.

PPM of Peaks

106.434

96.605

66.684

65.641

64.510

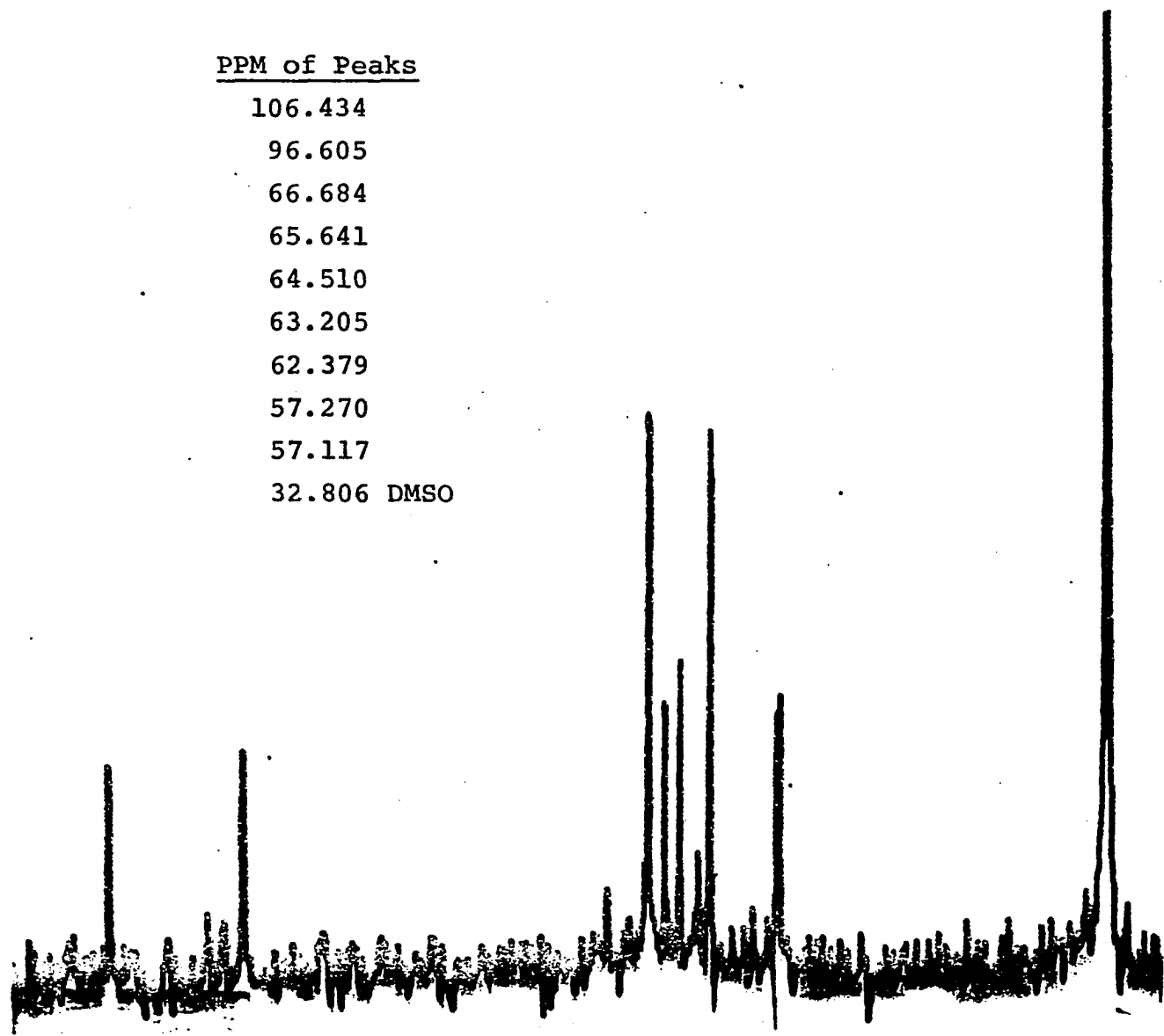
63.205

62.379

57.270

57.117

32.806 DMSO



levanicum were added. This mixture was layered with a small amount of toluene and incubated at room temperature for eight hours. The cells were removed by centrifuging and the aqueous phase was separated from the toluene and absorbed onto a charcoal celite mixture from which xylsucrose was eluted using a discontinuous step-wise ethanol-water gradient. Paper chromatography (pyridine:ethylacetate:water 10:4:3 v/v) of the acid hydrolysis products of the xylsucrose fraction showed the existence of xylose and fructose as the only products resulting from this reaction.

3. Synthesis of 6,6'-dichloro-6,6'-dideoxysucrose

The synthesis of the title compound was carried out according to the method of Anisuzzamen and Whistler (22). This involved the use of triphenylphosphine and carbon tetrachloride to carry out the direct conversion of the 6, and 6' hydroxyl groups into chloro groups.

Sucrose (3.4 g, 10 mM) was dissolved in refluxing dry pyridine (340 ml). Upon cooling to 0°C, one half of this solution was treated with triphenylphosphine (7.8 g, 30 mM) with constant stirring and cooling.

The resulting solution containing the sucrose and triphenylphosphine was stirred and cooled while carbon tetrachloride (1.4 ml, 15 mM) was added dropwise. The temperature was then raised to 70°C and maintained at this

point with constant stirring of the reaction mixture in an oil bath for two hours. Methanol (30 ml) was added to the reaction mixture at the end of the two-hour period and the resultant mixture was concentrated to a syrup on a rotary evaporator. Residual pyridine was removed by adding toluene to form an azeotrope which was removed by evaporation on the rotary evaporator again.

Purification of the 6,6'-dichloro-6,6'-dideoxysucrose was carried out by silica gel column chromatography using ethylacetate:ethanol:water (45:5:3 v/v/v) as the eluent. The appropriate fractions as determined by TLC (ethylacetate:ethanol:water 45:5:3 v/v/v) were combined and evaporated to dryness to yield purified 6,6'-dichloro-6,6'-dideoxysucrose. The reaction sequence is shown in Figure 14 and a ^{13}C -NMR spectrum of the product is shown in Figure 15.

4. Synthesis of 6,6'-dibromo-6,6'-dideoxysucrose

The synthesis of the title compound was carried out again according to the method described earlier for the dichloro analogue of sucrose. The reaction pathway is shown in Figure 16 and the ^{13}C -NMR spectrum of the acetylated product is shown in Figure 17. Sucrose (1.7 g, 5.0 mM) was dissolved in anhydrous refluxing pyridine (170 ml). Upon dissolution, the mixture was cooled to 0°C and triphenylphosphine (7.8 g, 30 mM) was added with constant stirring and cooling. The re-

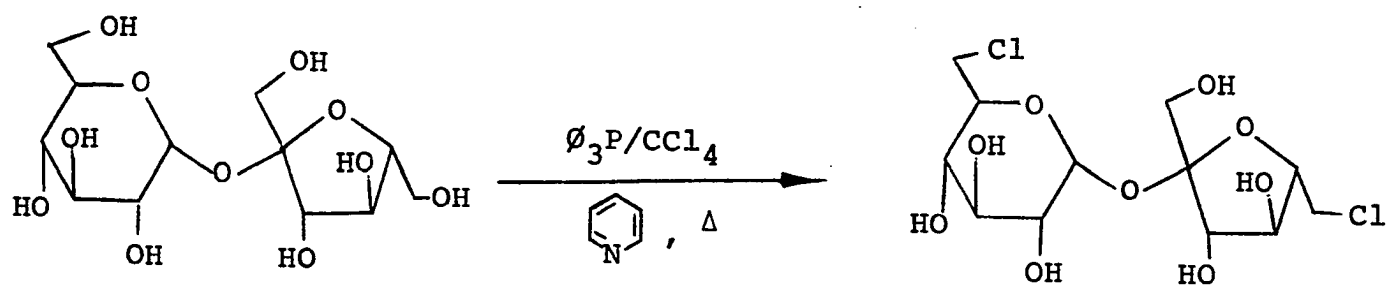


Figure 14. Synthesis of 6,6'-dichloro-6,6'-dideoxysucrose.

Figure 15. ^{13}C -NMR spectrum of 6,6'-dichloro-6,6'-dideoxysucrose.

PPM of Peaks

105.19

93.60

81.95

77.84

77.08

73.56

72.96

72.31

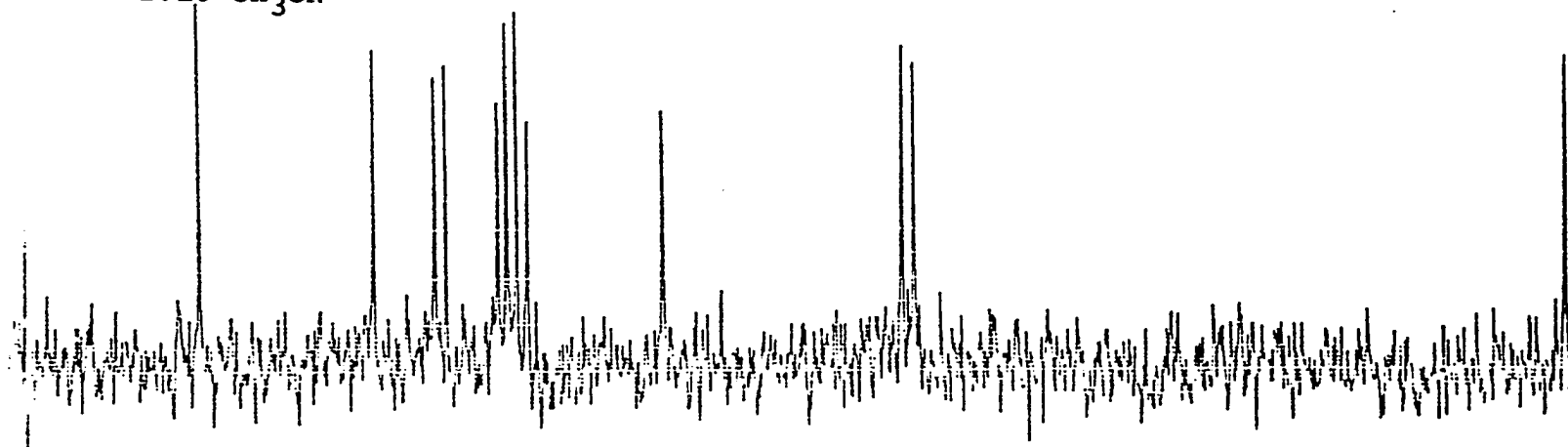
71.55

62.61

46.52

45.76

2.10 CH₃CN



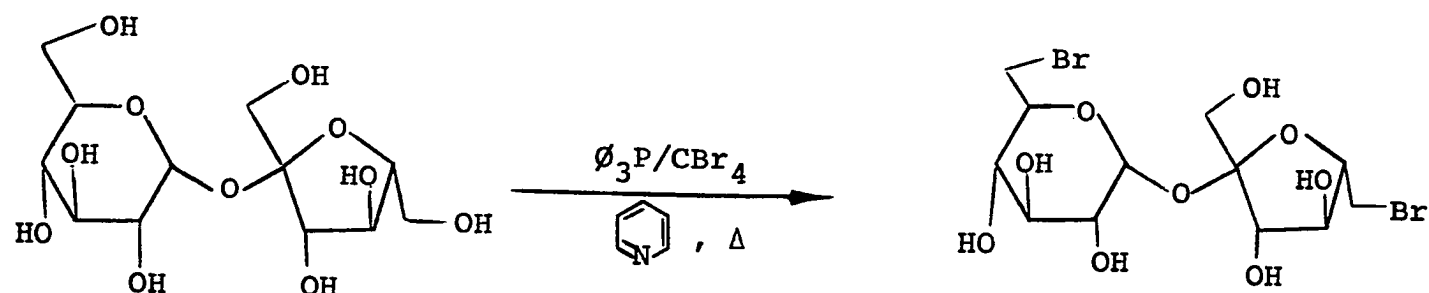
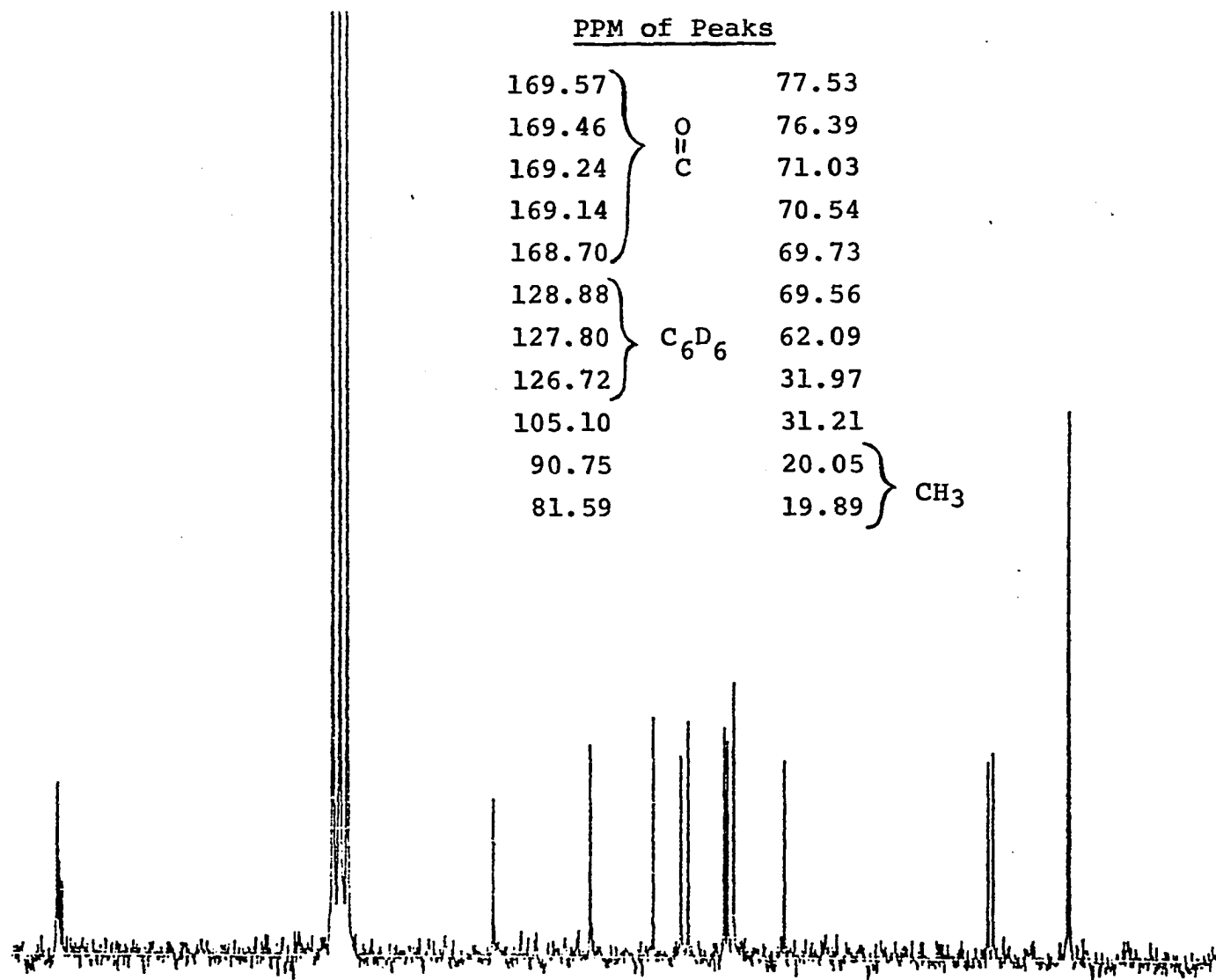


Figure 16. Synthesis of 6,6'-dibromo-6,6'-dideoxysucrose.

Figure 17. ^{13}C -NMR spectrum of 6,6'-dibromo-6,6'-dideoxy-2,3,4,3',4'-hexa-O-acetylsucrose.



sulting solution was stirred and cooled while carbon tetrabromide (5.0 g, 15 mM) was added. The temperature was then raised to 70°C and maintained at this point for two hours with constant stirring. Methanol was added to the reaction mixture as described before for the dichloro analogue and the resulting mixture was concentrated on a rotovap. Residual pyridine was removed by azeotrope formation with toluene and the resultant gummy mixture was applied to a column of silica gel. Elution of the column with ethylacetate: ethanol:water 45:5:3 (v/v/v) and collection of the appropriate fractions, as determined by TLC, gave pure 6,6'-dibromo-6,6'-dideoxysucrose (1.0 g, 2.1 mM) 42.3% yield.

5. Synthesis of 6,6'-dideoxy-6,6'-difluorosucrose

The synthesis of the title compound is outlined in Figure 18. 6,1',6'-Tri-O-tritylsucrose (2) was synthesized according to the method of Hough, et al. (35). Sucrose (75.0 g, 0.22 moles) was dissolved in pyridine (1.1 l) by stirring and refluxing for thirty minutes and then cooled to 0°C. A solution of triphenylchloromethane (207.4 g, 0.74 moles) in pyridine (250 ml) was added dropwise to the sucrose solution with continuous stirring and cooling. The resultant solution was stirred at ambient temperature (~23°C) for forty-eight hours; it was then concentrated under reduced pressure to a thick syrup which was subsequently

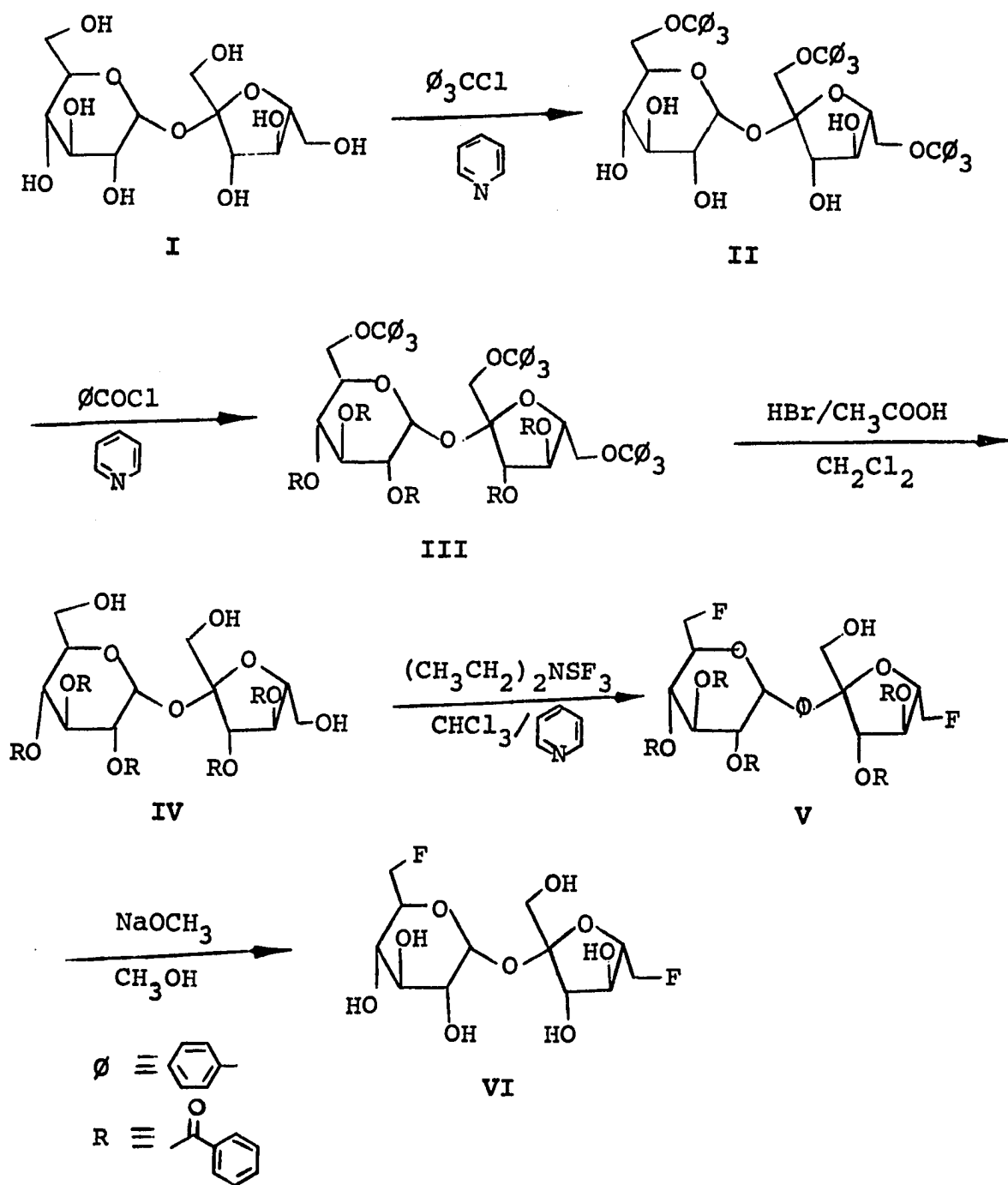


Figure 18. Synthesis of 6,6'-dideoxy-6,6'-difluorosucrose.

dissolved in chloroform (1 l) and washed with aqueous 1 N HCl (1 l) followed by a saturated sodium bicarbonate wash (aqueous, 1 l) and a final wash with distilled water (1 l). The organic layer was dried over anhydrous calcium sulfate, filtered, evaporated to a syrup and dissolved in toluene. The toluene solution was evaporated under vacuo to remove residual pyridine. The resultant product was a thick, white, foam-like material. Additional product could be recovered by extracting the aqueous washings with diethylether and repeating the procedure described. The total weight of the product amounted to 270.6 g.

Analysis of the product by TLC (Methanol:acetone:water:chloroform 20:20:3:57 v/v) (20) showed it to be a mixture of tritanol, 6-mono-0-tritylsucrose, 6'-mono-0-tritylsucrose, 6,6'-di-0-tritylsucrose, 6,1'-di-0-tritylsucrose, 1',6'-di-0-tritylsucrose, 6,1',6'-tri-0-tritylsucrose, unreacted sucrose, and a small amount of unidentified material, probably higher substitutions of the sucrose molecule (see Figure 18).

Purification of 6,1',6'-tri-0-tritylsucrose from the reaction mixture was carried out by first dissolving the syrupy reaction mixture in ether, absorbing the solution onto silica gel and applying this onto a dry packed column of silica gel. The column was first eluted with chloroform (1l) which removed the tritanol, followed by an elution with a

mixture of chloroform:acetone (8:1 v/v) (11). Appropriate fractions were pooled, as determined by TLC analysis, to obtain purified 6,1',6'-tri-0-tritylsucrose (122.3 g, 52% yield). TLC analysis indicated the presence of only one component (see Figure 18).

a. 6,1', 6'-Tri-0-trityl-2,3,4,3',4'-penta-0-benzoyl-sucrose (3) Compound (2) (41.3 g, 38.6 mM) was dissolved in anhydrous pyridine (250 ml). Benzoyl chloride (51.6 ml, 444.1 mM) was added dropwise with constant stirring and cooling. The reaction mixture was stirred for twenty-four hours at room temperature and then poured into ice-water. The resultant gummy precipitate was dissolved in methylene chloride, washed with water, and evaporated to a syrup under reduced pressure. The resultant amorphous material was dissolved in toluene and evaporated to a solid in order to remove residual pyridine. The solid was dissolved in benzene and applied to a column of silica gel in the same manner as described above. The column was eluted with benzene:ether (18:1 v/v) and the appropriate fractions, as determined by TLC analysis, were collected, pooled, and concentrated to yield 73.4 g (46.2 mM, 40.4%) of 6,1',6'-tri-0-trityl-2,3,4,3',4'-penta-D-benzoylsucrose.

b. 2,3,4,3',4'-Penta-0-benzoylsucrose (4) Compound (3) (23.5 g, 15.4 mM) was dissolved in methylene chloride (150 ml) cooled to 0°C using an ethanol-ice bath and mixed with

glacial acetic acid (150 ml) with constant stirring and cooling. A solution of hydrogen bromide (32%) in glacial acetic acid (15 ml) was added to the above solution. Stirring and cooling were maintained for eight minutes after the addition of the hydrogen bromide solution. The reaction was quenched by pouring the mixture into a saturated (1 l) aqueous solution of sodium acetate; the organic layer was collected and washed with a saturated aqueous solution of sodium bicarbonate (1 l) until all effervescence ceased. The methylene chloride layer was evaporated and dried over anhydrous calcium sulfate. TLC (benzene:ether (18:1 v/v) indicated that the detritylation was essentially complete. The product, 2,3,4,3',4'-penta-O-benzoylsucrose (4) was purified by fluorescent dry column chromatography by elution with benzene:ether (18:1 v/v). This procedure was repeated three more times to convert the remaining intermediate (3) into the detritylated product (4). The final yield was 8.5 g (9.8 mM, 24.3%).

c. 6,6'-Dideoxy-6,6'-difluoro-2,3,4,3'4'-penta-O-benzoyl sucrose (5) Intermediate (4) (8.5 g, 9.8 mM) was dissolved in chloroform (140 ml) and the resultant solution was added dropwise to a cooled (-10°C) solution of diethylaminosulfur trifluoride (15.5 ml, 124.0 mM) in a mixture of chloroform (140 ml) and pyridine (30 ml). The resulting reaction mixture was held at this temperature with constant stirring

for fifteen minutes and then raised to 60°C for one hour with constant stirring. The reaction was quenched by dilution with four volumes of chloroform and the subsequent addition to an equal volume of a saturated aqueous sodium bicarbonate solution with constant stirring until all effervescence ceased. The chloroform solution was separated from the aqueous layer, dried over anhydrous calcium sulfate, filtered and evaporated to a syrup. The syrup was dissolved in chloroform, absorbed onto silica gel, and applied to a column of dry packed silica gel. The column was eluted with chloroform and the appropriate fractions, as determined by TLC analysis (100% chloroform), were combined and concentrated to a glassy solid. The yield of (5) was 2.35 g (2.7 mM, 27.6%).

d. 6,6'-Dideoxy-6,6'-difluorosucrose (6) The fluorinated intermediate (5) was dissolved in methanol (340 ml) and a solution (3 ml) of sodium methoxide (1 N) was added and the reaction mixture was stirred overnight at ambient temperature. The reaction mixture was neutralized by adding Amberlite IRC-50 (H^+) resin, filtered and evaporated to a syrup. The syrup was extracted with a mixture of chloroform and water (1:1 v/v). The aqueous phase was separated, filtered and extracted with ethylacetate to remove more of the methylbenzoate. The aqueous phase was separated, filtered and evaporated under vacuo to a thin film. This was dissolved in a small amount of methanol and applied to a column of

silica gel, which was eluted with ethylacetate:ethanol:water (45:5:3 v/v). The appropriate fractions (TLC analysis ethylacetate:ethanol:water 45:5:3 v/v) were collected, pooled, evaporated to dryness in vacuo and dissolved in a minimum amount of acetonitrile. Upon cooling to 0°C, crystalline 6,6'-dideoxy-6,6'-difluorosucrose precipitated out of solution. The yield of (6) was 200 mg (0.6 mM, 21.4%). The overall yield from sucrose was 0.3%. A ^{13}C -NMR and ^{19}F -NMR were obtained for this product and are shown in Figures 19 and 20.

6. Synthesis of 6-deoxy-6-fluorosucrose and 6'-deoxy-6'-fluorosucrose

The title compounds were prepared as a mixture in a manner identical to the preparation of 6,6'-dideoxy-6,6'-difluorosucrose with the exception that 6 and 6'-mono-0-tritylsucrose were used rather than 6,6'-di-0-tritylsucrose. The synthetic scheme is outlined in Figure 21.

a. 6-Mono-0-tritylsucrose (2) This was synthesized according to the method of Otake (20). Sucrose (1) (342.3 g, 1 mole) was dissolved in anhydrous pyridine (10 l) and tritylchloride (334 g, 1 mole) was added. The resulting reaction mixture was stirred for four days at ambient temperature. TLC analysis (methanol:acetone:water:chloroform 20:20:3:57 v/v) showed that the reaction mixture contained the same components as the one described earlier for the synthesis of the difluoro analogue of sucrose. The reaction mixture was concentrated

Figure 19. ^{13}C -NMR spectrum of 6,6'-dideoxy-
6,6'-difluorosucrose.

PPM of Peaks

105.57

93.98

88.56

87.86

81.57

81.07

80.76

80.43

78.16

78.05

74.59

74.21

73.34

72.24

72.90

70.36

70.03

62.88

2.1 CD₃CN center

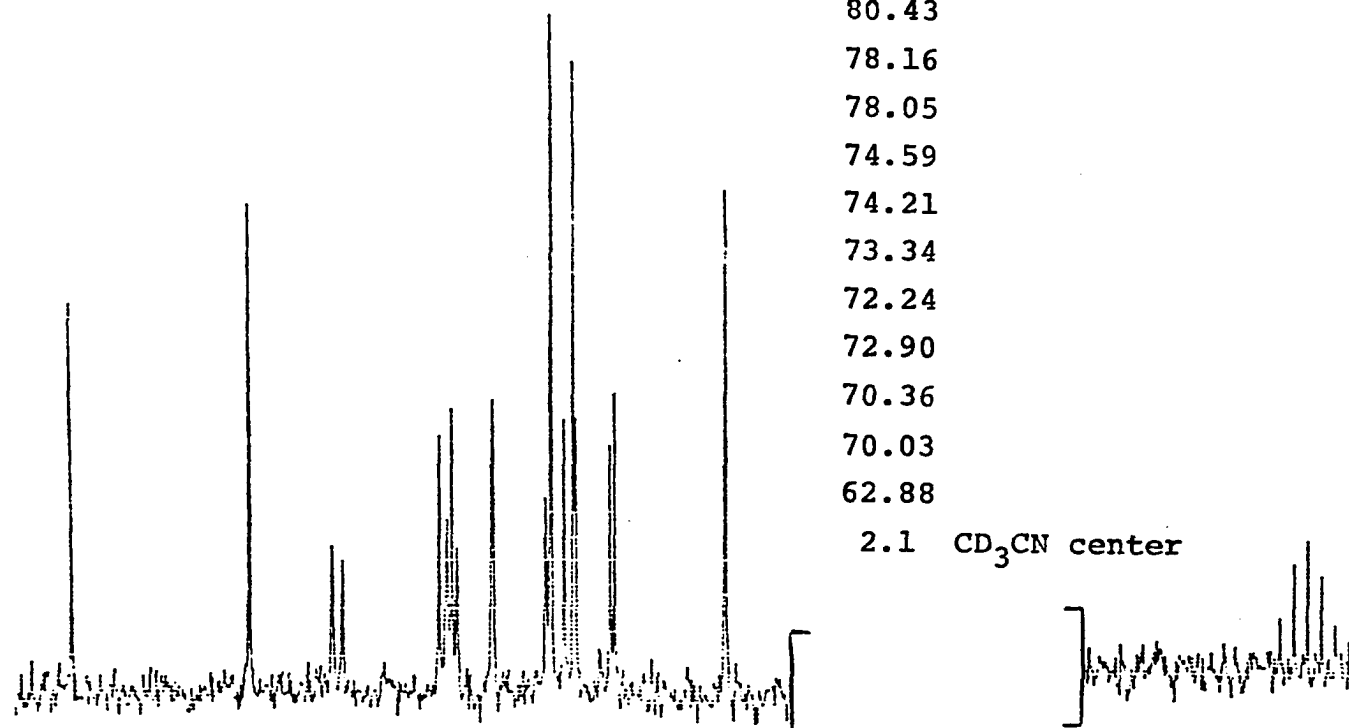


Figure 20. ^{19}F -NMR spectrum of 6,6'-dideoxy-
6,6'-difluorosucrose.

PPM of Peaks

222.804

223.092

223.381

223.633

223.921

224.174

PPM of Peaks

230.336

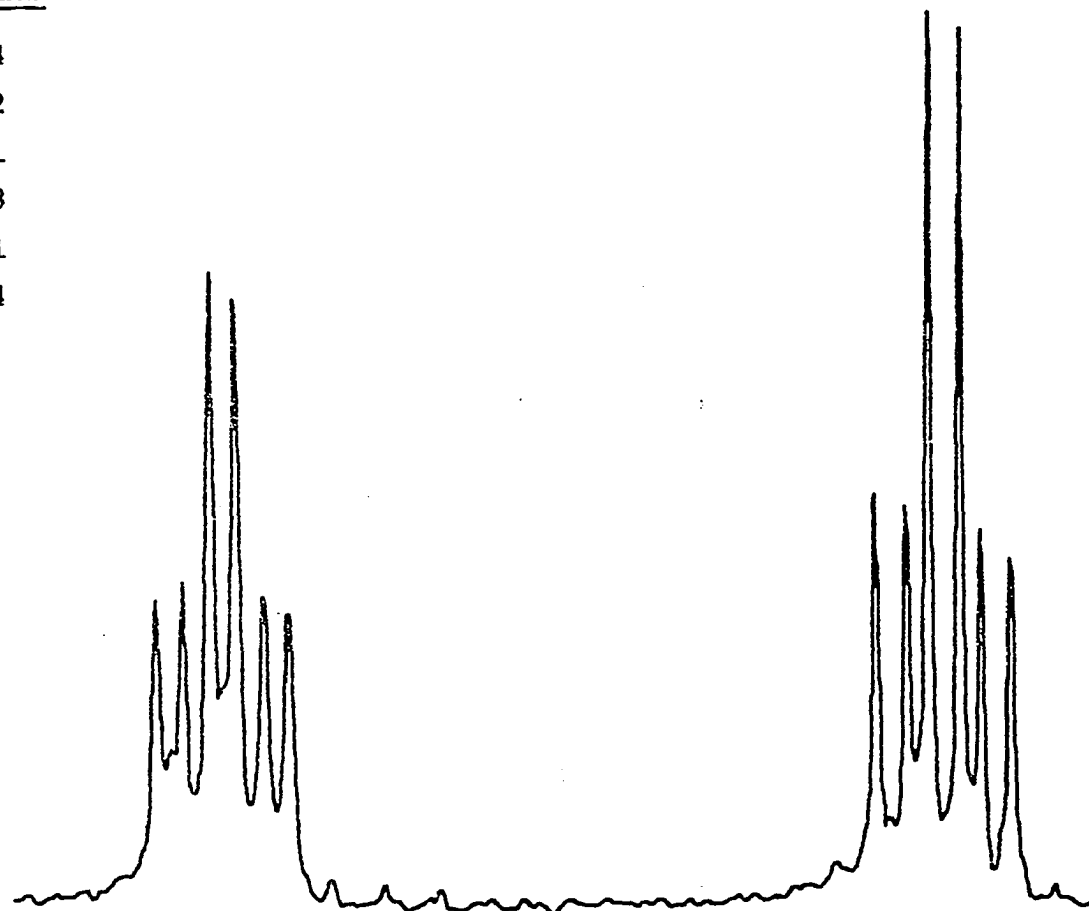
230.661

230.913

231.238

231.454

231.778



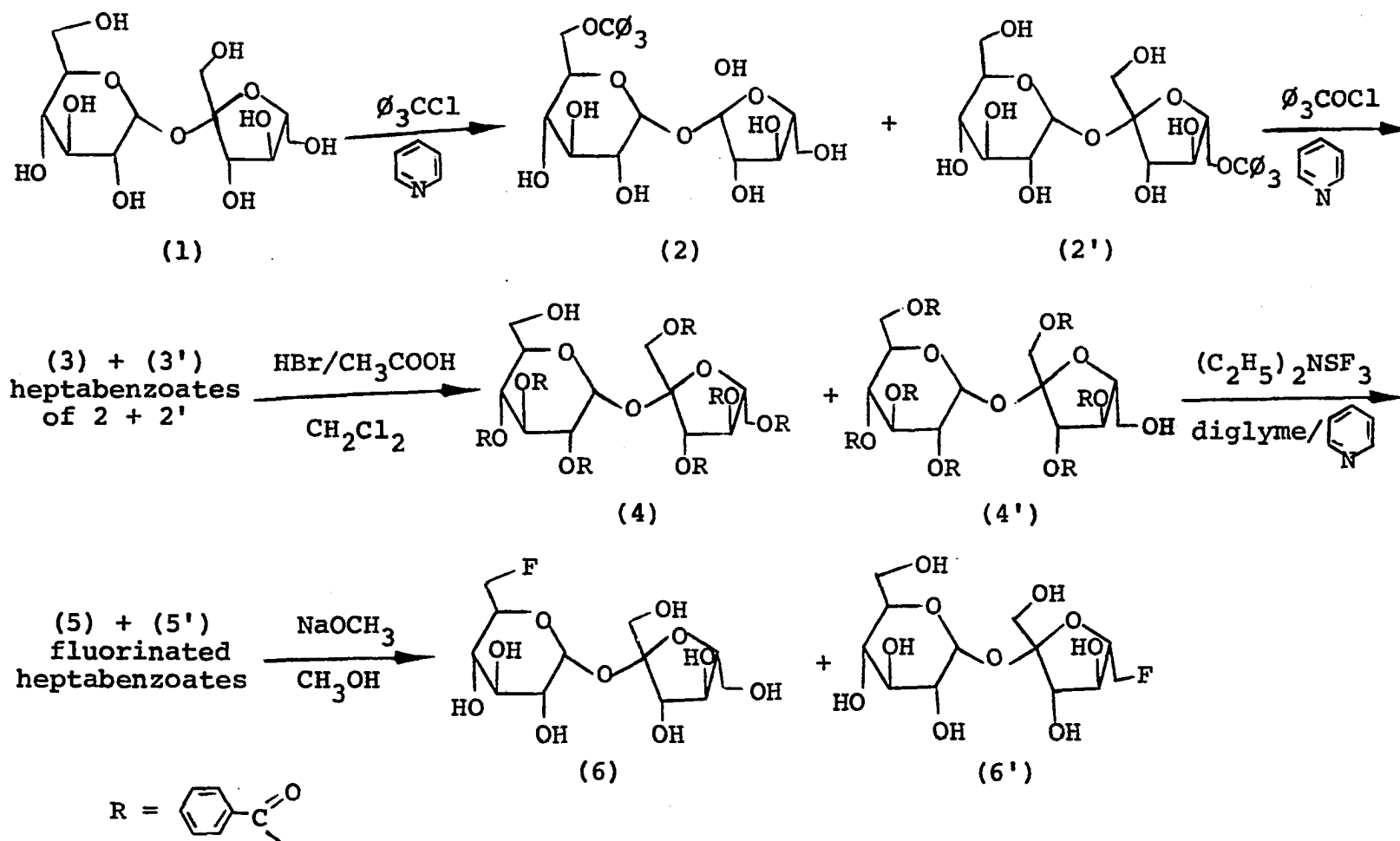


Figure 21. Synthesis of 6-deoxy-6-fluorosucrose and 6'-deoxy-6'-fluorosucrose.

under reduced pressure to a thick syrup which was dissolved in n-butanol (1ℓ). The butanol solution was washed with water and the organic layer was collected and dried over calcium sulfate. The solution was filtered and evaporated under reduced pressure to a very thick syrup which was dissolved in a minimum amount of methanol and the resulting solution slurried with an amount of silica gel sufficient to absorb all of the solution. The mixture was stirred well and allowed to air dry overnight. The silica gel with the absorbed product after drying was applied to a dry packed column of silica gel. Approximately 60 g of product in 230 ml of silica gel was applied to a column of 3 cm in diameter and 100 cm in height (or 707 ml of silica gel in a tightly packed dry column ~450 g). The column was selectively eluted in the following manner; first elution with methylene chloride which removes most of the tritanol (2ℓ), second elution with methylene chloride:acetone (8:1 v/v) which removes most of the tri-0-tritylsucrose, third elution with methylene chloride:acetone (1:1 v/v) which removes most of the di-0-tritylsucrose, and the fourth elution with methanol which elutes most of the mono-0-trityl sucrose. A mixture of 6-mono-0-tritylsucrose and 6'-mono-0-tritylsucrose results from this procedure. It is possible to separate 6-mono-0-tritylsucrose and 6'-mono-0-tritylsucrose by

repeated crystallization of 6-mono-0-tritylsucrose from the mixture. In addition, it is possible to remove most of the unreacted sucrose from the reaction mixture by triturating the syrupy mixture, prior to column chromatography, with acetone and removing the undissolved sucrose by filtration.

b. 6-Mono-0-trityl-2,3,4,1',3',4',6'-hepta-0-benzoyl-sucrose (3) Some of the resulting mixture of mono-0-tritylsucrose ethers (4.5 g, 7.7 mM) was dissolved in 50 ml of anhydrous pyridine. The resulting solution was cooled to 0°C and benzoyl chloride (14.4 ml, 124.0 mM) was added dropwise with constant stirring. The reaction mixture, after the addition of the benzoyl chloride, was allowed to warm up to room temperature and it was stirred at this temperature for twenty-four hours. The mixture was poured into ice water and the precipitate which formed was extracted into ethylacetate, which was subsequently washed with water, dried over anhydrous calcium sulfate, and concentrated to a thick syrup under vacuo. The syrup was applied to a column of silica gel and elution with chloroform was carried out. The total eluate was collected, combined, and concentrated to an amorphous solid. This solid (3.8 g, 2.9 mM) consisting of a mixture of 6-mono-0-trityl-2,3,4,1',3',4',6'-hepta-0-benzoylsucrose and 6'-mono-0-trityl-2,3,4,6,1',3',4'-hepta-0-benzoylsucrose was dissolved in methylene chloride (25.0 ml) and the solution was cooled to -15°C using an

acetone/ice bath. Glacial acetic acid (24 ml) was added with constant stirring and cooling and ten minutes later 2.4 ml of a 32% (w/w) solution of hydrogen bromide in glacial acetic acid was added with constant stirring and cooling maintained for eight minutes after the addition of the hydrogen bromide solution.

The detritylation was quenched by dilution using ~48 ml of methylene chloride, followed by an aqueous sodium acetate (400 g/1.5 l) wash which decolorized the methylene chloride solution. The organic layer was separated and treated with an aqueous solution of sodium bicarbonate (saturated 1l total volume). Additional bicarbonate was added until all effervescence ceased. The organic layer was then separated, dried, and evaporated, under reduced pressure, to a syrup. TLC analysis (benzene:ether 9:1 v/v and 100% chloroform) indicated the presence of tritanol and two other reaction products, namely 2,3,4,1',3',4',6'-hepta-0-benzoylsucrose and 2,3,4,6,1',3',4'-hepta-0-benzoylsucrose. This mixture was applied to a column of silica gel and eluted with chloroform to remove the tritanol from the mixture which eluted from the column first. The two heptabenzoates were collected from the eluate (TLC analysis) as a mixture and concentrated to a glass-like material. This mixture (2.37 g, 2.21 mM) was dissolved in diglyme (5 ml) which had previously been stored over molecular sieves for two days,

and was added dropwise with constant stirring to a solution of diethylaminosulfur trifluoride (1.0 ml, 1.43 g, 8.85 mM) in diglyme (5 ml) and pyridine (2.7 ml) previously cooled to -15° to -20°C using an ethanol-ice bath. The reaction mixture was stirred for fifteen minutes at this temperature; then the cooling bath was removed and the reaction mixture allowed to reach 23°C . It was then placed in an oil bath and heated to 65°C for three hours with constant stirring. The reaction mixture was then cooled to room temperature and poured into ice-water (100 ml). The product precipitated out of solution and was collected by filtration and taken up in ethylacetate. The ethylacetate was washed with water and evaporated to yield a white foam (solid). The foam was dissolved in a small amount of chloroform and added to a column (3 cm diameter and 45 cm length) of silica gel which had been slurried in chloroform. The column was eluted with chloroform:methanol (10:1 v/v) and the elution was monitored by TLC (100% chloroform) which indicated that the product had eluted from the column in the early fractions. The appropriate fractions were combined and concentrated to a foam.

Approximately 1.0 g of this was dissolved in 130 ml of methanol to which was then added 1.3 ml of a 1 M sodium methoxide solution. The reaction mixture was stirred overnight and then treated with Amberlite IRC-50 (H^{+}) ion

exchange resin to neutralize the reaction mixture and remove the sodium ions. The neutralization was monitored by pH paper. The resin was removed by filtration and the filtrate evaporated to a glassy solid. Final yield ~50%.

A ^{13}C -NMR spectrum of the products is shown in Figure 22 and an ^{19}F -NMR spectrum is shown in Figure 23.

7. Inhibitors derived from tritripsylosucrose

The reaction sequence for this preparation is shown in Figure 24.

a. 6,1',6'-tri-0-(2,4,6-triisopropylbenzenesulfonyl)-sucrose (I) Tritripsylosucrose (I) was prepared according to the method described by Almquist and Reist (21).

Sucrose (52 g) was dissolved in anhydrous pyridine (750 ml) by refluxing vigorously for approximately one hour. The resulting solution was cooled to 0°C and then treated with 2,4,6-triisopropylbenzenesulfonyl chloride (231.5 g). The cooling bath was removed and the resulting mixture was allowed to stand for four days at ambient temperature (25 - 28°C). The reaction mixture was then cooled to 0°C again and distilled water (125 ml) was added dropwise with constant stirring. The resulting mixture was stirred for thirty minutes and then concentrated to a thick yellow syrup by evaporation under reduced pressure at 50°C. The syrup was slurried with ethylacetate, cooled to 0°C and

Figure 22. ^{13}C -NMR spectrum of the mixture of
6-deoxy-6-fluorosucrose and 6'-deoxy-
6'-fluorosucrose.

PPM of Peaks

105.14	73.83	61.53
104.92	73.66	58.66
93.55	72.91	
93.33	72.36	
87.37	72.20	
82.60	70.63	
80.87	69.87	
80.27	69.55	
79.95	63.80	
77.67	62.67	
75.34	62.39	
74.15		

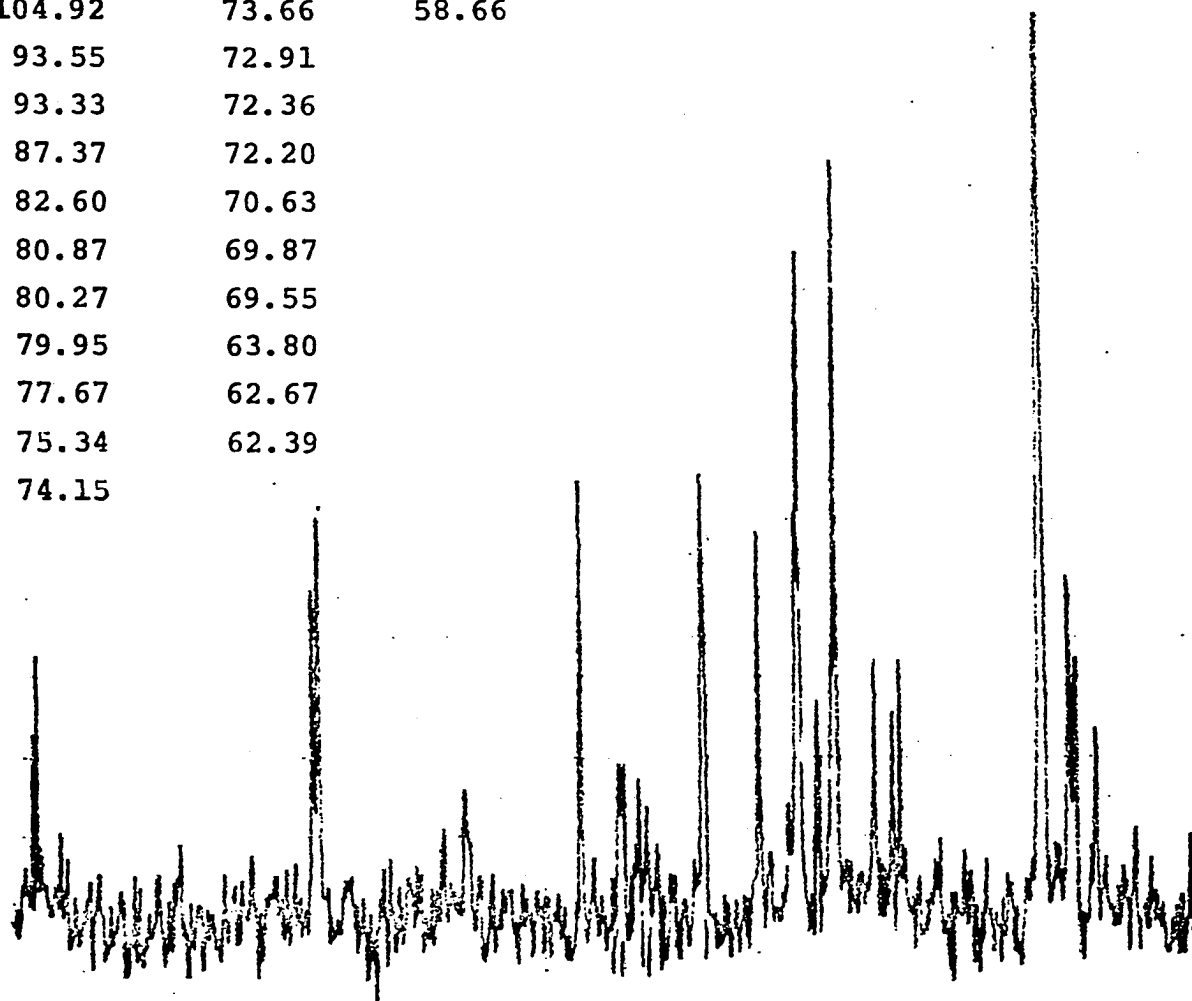
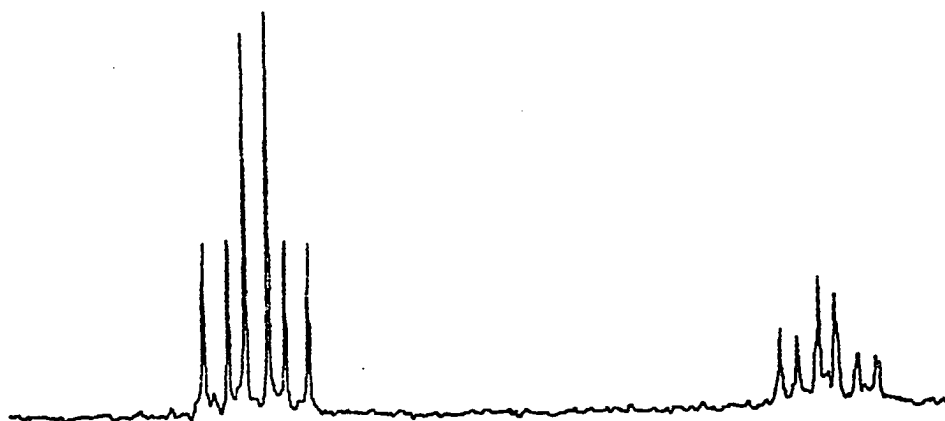
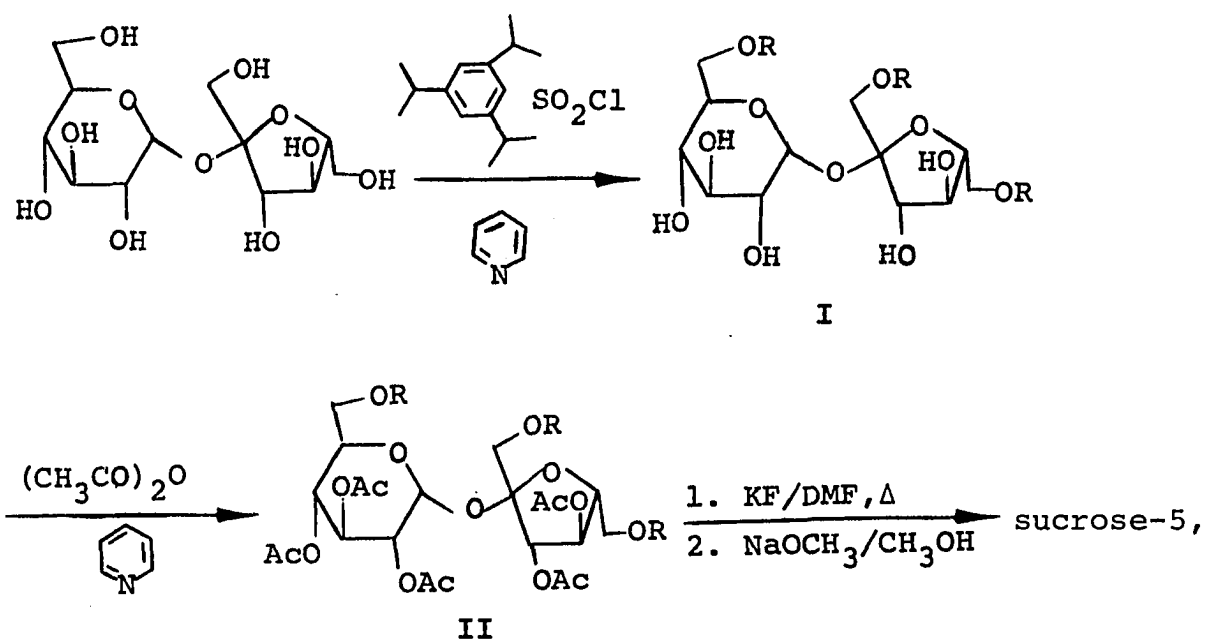


Figure 23. ^{19}F -NMR spectrum of the mixture of 6-deoxy-6-fluorosucrose and 6'-deoxy-6'-fluorosucrose.

PPM of Peaks

115.400
115.725
115.958
116.284
116.516
116.842
123.236
123.468
123.794
124.026
124.352
124.585





5'-diene + 3,6-anhydrosucrose + 3,6,1',4'-dianhydrosucrose
 + 6,1',6'=trideoxy-6,1',6'-549fluorosucrose + partially
 sulfonated derivatives.

Figure 24. Reaction sequence leading to inhibitors from tri-O-tripsylsucrose.

filtered. The filter cake was washed with cold ethylacetate. The ethylacetate solutions were combined and washed successively with cold 2N hydrochloric acid, saturated sodium bicarbonate, 4% aqueous sodium chloride and finally distilled water. The ethylacetate layer was dried over anhydrous calcium sulfate, filtered, and evaporated under vacuo to dryness resulting in a thick white foam (I). Comparison of the ^{13}C NMR spectrum of (I) with that obtained for the title compound by Almquist and Reist (27) indicates that they are identical (see Table 1).

Table 1. Comparison of chemical shifts of tri-0-tripsylsucrose.

Tri-0-tripsylsucrose	Compound I
103.3	100.839
92.9	90.541
81.9	78.618
77.5	74.980
75.4	72.803
74.2	71.666
72.2	69.717
71.6	69.197
71.3	68.515
71.0	67.670
69.8	67.020
67.0	64.519

b. 6,1',6'-Tri-O-(2,4,6-triisopropylbenzenesulfonyl)-sucrose-2,3,4,3',4'-penta-acetate (II) The tritriipsyl-sucrose (I) was dissolved in pyridine (200 ml) and acetic anhydride (160 ml) was added slowly with stirring and cooling (4°C). The cooling bath was removed after the addition of the acetic anhydride had been completed and the mixture was allowed to reach ambient temperature (24-28°C). It was stirred at this temperature for twenty-four hours at the end of which the reaction mixture was poured into ice-water resulting in the formation of a thick gummy precipitate. The precipitate was dissolved in ethylacetate, washed with cold 2N hydrochloric acid, followed by a saturated solution of sodium carbonate, and finally distilled water. The ethylacetate solution was dried over anhydrous sodium sulfate then filtered and evaporated to dryness under vacuo at ~50°C to yield the pentaacetate (II).

c. Treatment of (II) with potassium fluoride Compound (II) was dissolved in anhydrous dimethylformamide (250 ml) to which was added anhydrous potassium fluoride (90.0 g). The resulting mixture was refluxed for twenty-four hours and then poured into crushed ice. The precipitate which formed was taken up in ethylacetate, washed with a 4% aqueous sodium chloride solution and then reduced in volume to a thick syrup. This syrup was dissolved in a few milli-

liters of dry methanol and enough 1M sodium methoxide solution was added to bring the final concentration in the reaction mixture to 0.03 M. This solution was allowed to stand overnight (i.e., 12 hours) at room temperature and then it was absorbed onto 600 g of charcoal which was subsequently washed with three liters of distilled water followed by one liter of a 10% t-butyl alcohol solution (v/v) in water. The t-butyl alcohol solution which eluted the carbohydrate was concentrated under vacuo to a glass-like material. Thin layer chromatography indicated the presence of at least seven components which could not be easily resolved either by TLC or paper chromatography.

An aqueous solution of this material (~27 mg/ml) was the same pH as the buffer used, i.e., pH 5.5.

8. Enzyme assays

Dextranucrase was assayed using a method similar to that employed by Germaine, et al. (36).

A typical example of this assay is as follows: dextranucrase enzyme, appropriately diluted (dilution factor determined experimentally) in a 20 mM pyridine-acetate buffer, (25 μ l) was mixed with 20 mM pyridine-acetate buffer (25 μ l, pH 5.5) and incubated at ~25°C for ten minutes. 14 C-Sucrose (150 mM) in 20 mM pyridine -acetate buffer (25 μ l) was added and aliquots (10 μ l) were withdrawn at 3, 6, 9, 12 and 15 minutes after addition of the 14 C-sucrose solution.

Each aliquot was spotted on a piece of Whatman 3MM paper 1.5 x 1.5 cm which was immediately dropped into a beaker of absolute methanol and stirred for fifteen minutes. The methanol solution was changed twice every fifteen minutes in order that the paper squares be washed three times with the solution. This procedure removed the low molecular weight materials including fructose and any unreacted sucrose and in addition it precipitates any polysaccharide which formed during the reaction onto the paper.

Upon completion of the third wash the papers were dried, placed in a toluene scintillation cocktail and the total counts obtained from a scintillation counter. The inhibitors were assayed at the same time in an identical manner except that the inhibitor at the appropriate concentration was dissolved in the pyridine-acetate buffer which was added to the enzyme solution and incubated for ten minutes prior to the addition of the ^{14}C -sucrose solution.

The vials were counted up to 1 percent rsd.

III. RESULTS AND DISCUSSION

Based upon the proposed mechanism of action for dextranucrase (see Figure 4, p. 11), the possibility that the two nucleophilic groups X_1 and X_2 might be lipid pyrophosphate seemed very likely. This idea was tested in a somewhat indirect manner, namely by the use of a material which is known to bind tightly to groups such as dolichol or bactoprenol phosphates and pyrophosphates. The polypeptide antibiotic bacitracin, really a mixture of bacitracins elaborated by Bacillus subtilis and Bacillus licheniformis was demonstrated by Storm and Strominger (37) to exert its antibiotic effects by binding the bactoprenol pyrophosphate units in the enzymatic synthesis of the Salmonella O-antigen oligosaccharides.

The first assay, testing bacitracin for any inhibition towards dextranucrase, was done utilizing a sample of enzyme and following the reaction by measuring the amount of fructose released per unit time as a function of total reducing values on the AutoAnalyzer. The results of this assay are shown in Figure 25. This experiment demonstrated some inhibitory activity, i.e., 29.6% of bacitracin towards dextranucrase, however, there were several problems which had to be dealt with prior to repeating this type of an assay again. First of all the AutoAnalyzer assay measured

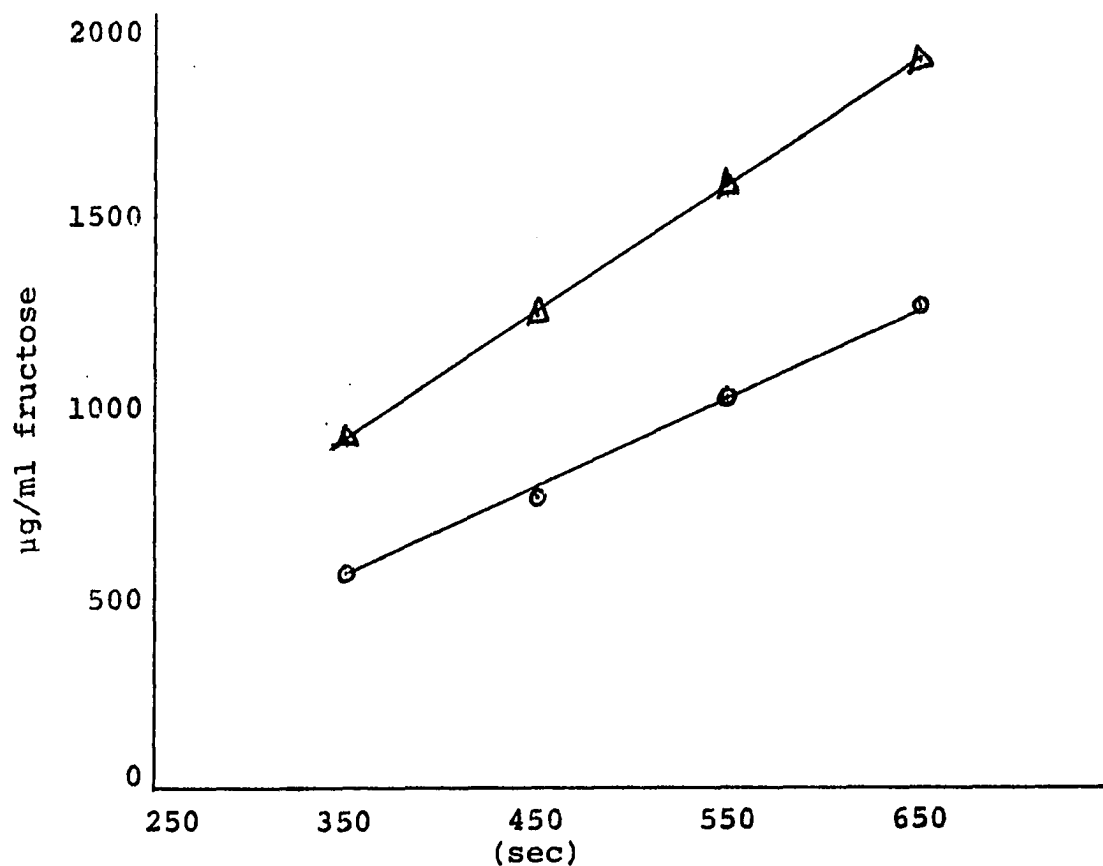


Figure 25. Assay of dextransucrase using bacitracin at 50 µg/ml (35 mM) as an inhibitor.

Δ = control

○ = bacitracin treated

% Inhibition = 29.6

NOTE: All values for % Inhibition are calculated from the equation

$$\%I = \frac{S_1 - S_2}{S_1} \times 100$$

%I = % Inhibition,

S_1 = slope of control

S_2 = slope of inhibitor treated enzyme

total reducing carbohydrates produced per unit time regardless of the chemical identity. In this case fructose from the reaction would be detected along with any small oligosaccharides and any glucose and additional fructose produced by invertase, dextranase, or levansucrase enzymes present in the mixture.

Due to these facts this experiment was repeated, using a more accurate measure of polysaccharide produced per unit time. This was done with the radio-labeled sucrose assay described earlier in the methods section. The results of this assay are shown in Figure 26. This assay at 250 $\mu\text{g/ml}$ of bacitracin showed 82% inhibition resulting from the bacitracin.

Further work with bacitracin was carried out and the same assay was repeated with bacitracin at approximately 54 mM yielding an inhibition of dextransucrase amounting to 93% as depicted in Figure 27.

Subsequent to this assay a similar assay was performed using the same concentration of substrate and bacitracin with the enzyme mutansucrase from S. mutans OMZ 176. This resulted in an inhibition of 83% of the enzymatic activity as shown in Figure 28.

Bacitracin, as it was stated earlier, is a mixture of very similar polypeptides which can be purified by ion

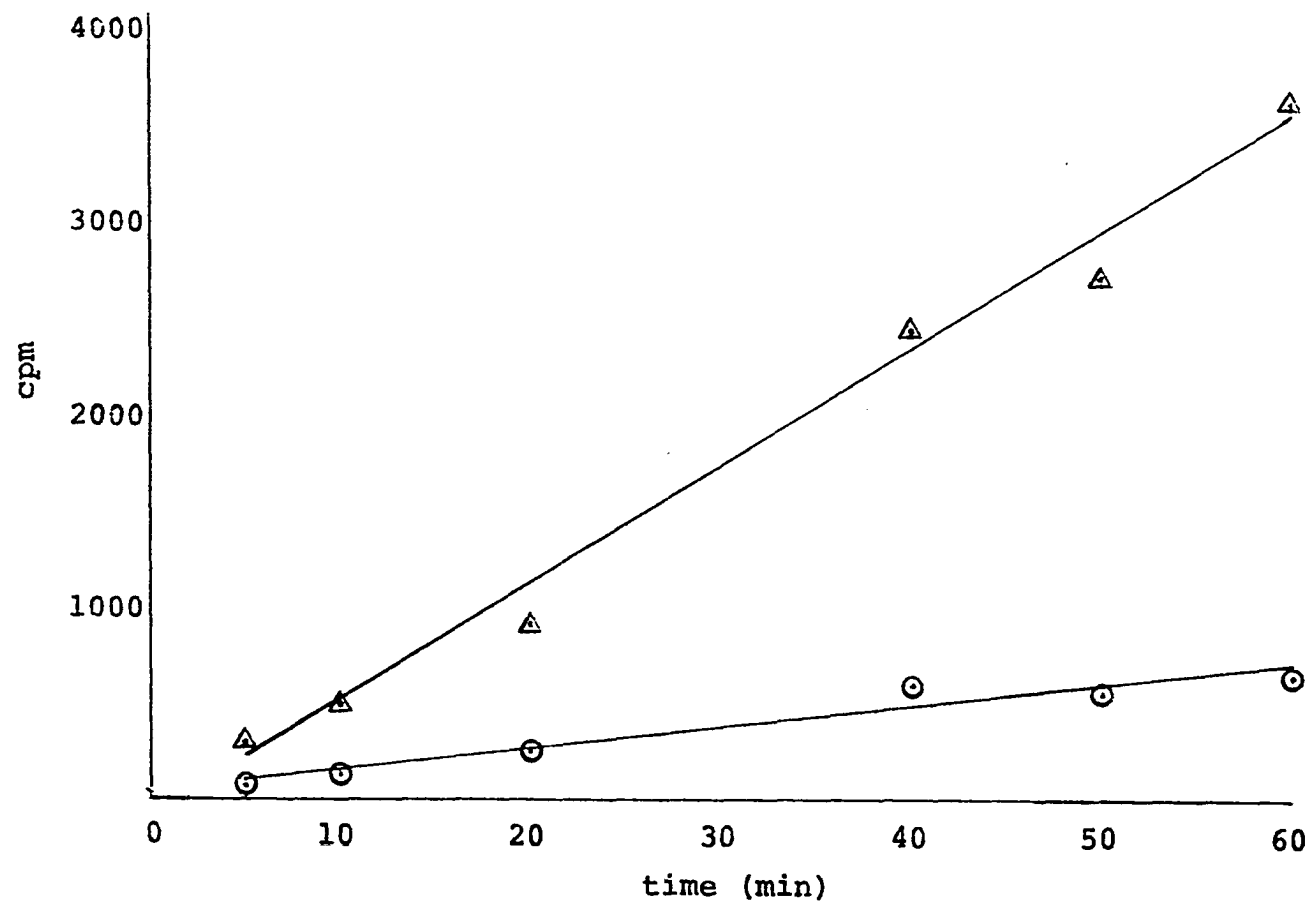


Figure 26. Assay of dextransucrase using bacitracin at 250 $\mu\text{g/ml}$ (177 mM) as an inhibitor.

Δ = control

\bigcirc = bacitracin

%I = 82.4

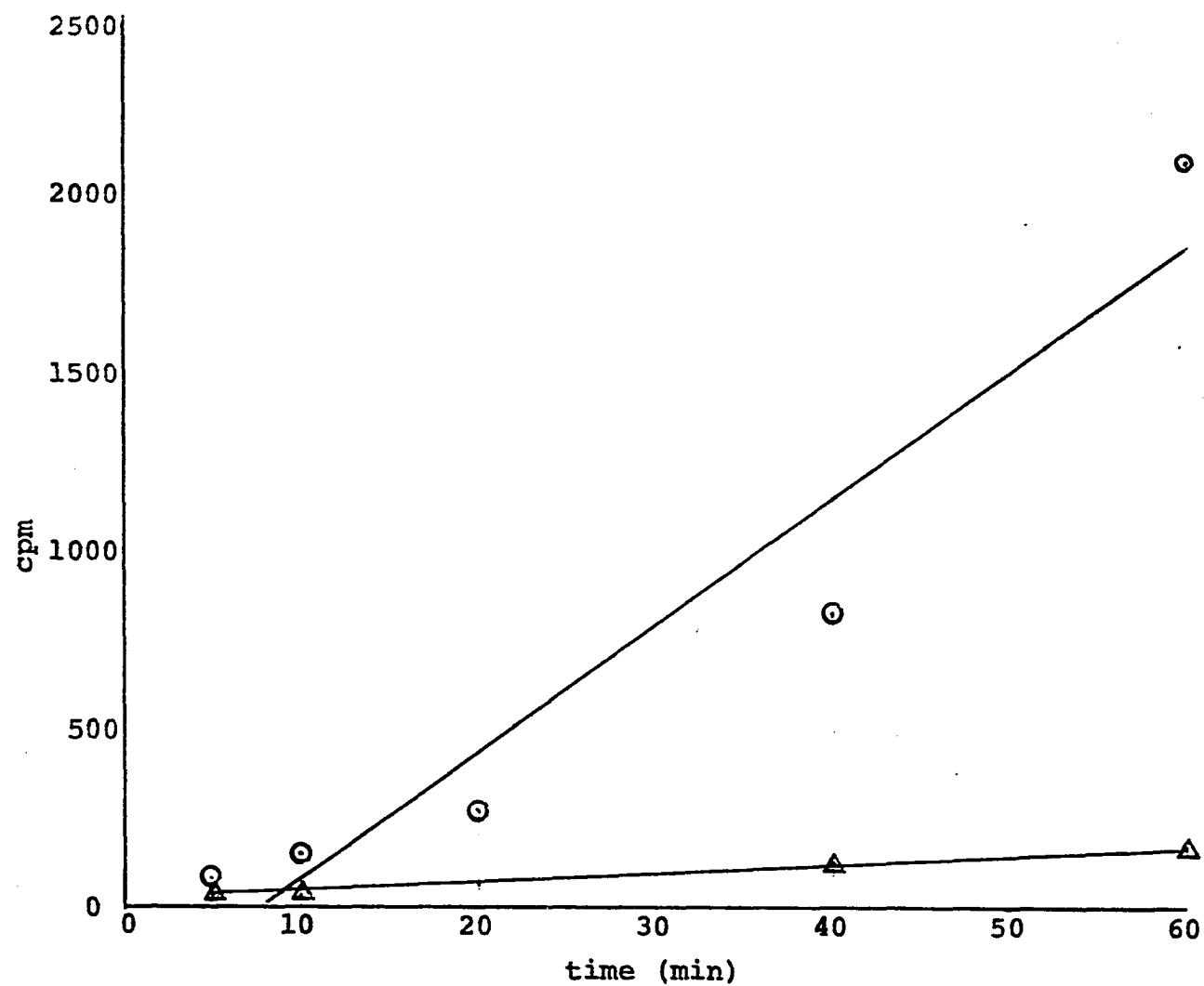


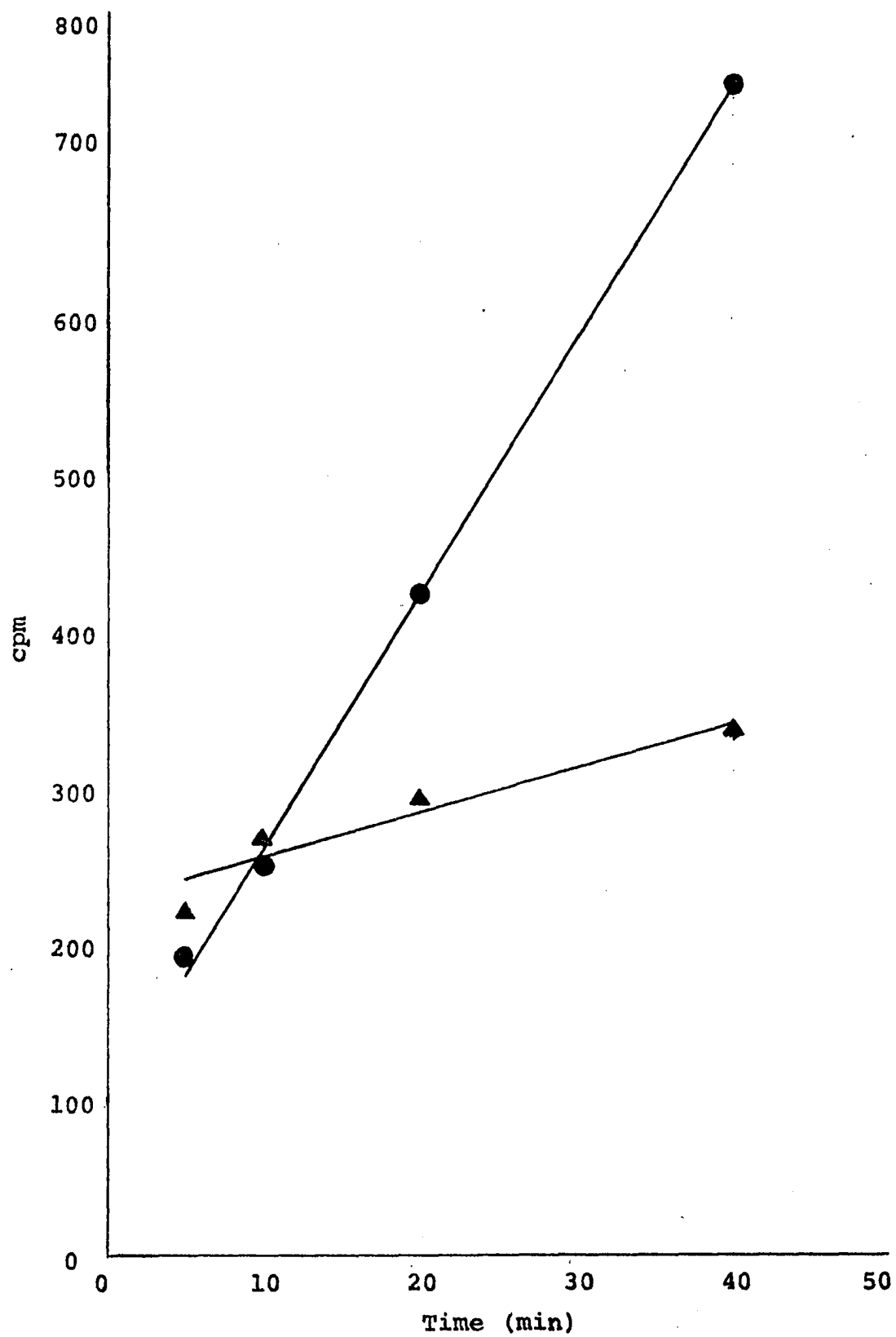
Figure 27. Assay of dextransucrase using as an inhibitor bacitracin at 54 mM.
 ○ = control Δ = bacitracin %I = 100

Figure 28. Assay of mutansucrase treated with and without bacitracin at 50 mM.

● = control

▲ = bacitracin

%I = 82.6



exchange chromatography. Such a purification was carried out and the results are shown in Figure 29.

Each peak I, II, III, and IV was treated with pyridine-acetate buffer pH = 5.0 and filtered on a membrane filter then each fraction was lyophilized and the resultant solid was assayed with dextransucrase to check for inhibition. The results of this experiment are shown in Figure 30.

The results of this assay indicate that peak IV which corresponds to a minor bacitracin component is the most potent inhibitor of the group.

Commercial bacitracin contains zinc and it is known that zinc can complex with lipid pyrophosphate units (37). Due to these reasons zinc was assayed as a potential inhibitor for dextransucrase and the results are shown in Figure 31.

These results indicate that zinc is a very potent inhibitor of dextransucrase. Later work by Dr. Walseth confirmed this experimental finding (38).

Initial attempts at clarification of the mechanism of action of the dextransucrase through the use of substrate analogues, involved the synthesis of α -D-xylopyranosyl- β -D-fructofuranoside (xylsucrose). The synthesis of xylsucrose was carried out according to the method of Avigad et al. (34) which involved the levansucrase catalyzed transfructosylation reaction using raffinose as a fructofuranosyl donor and

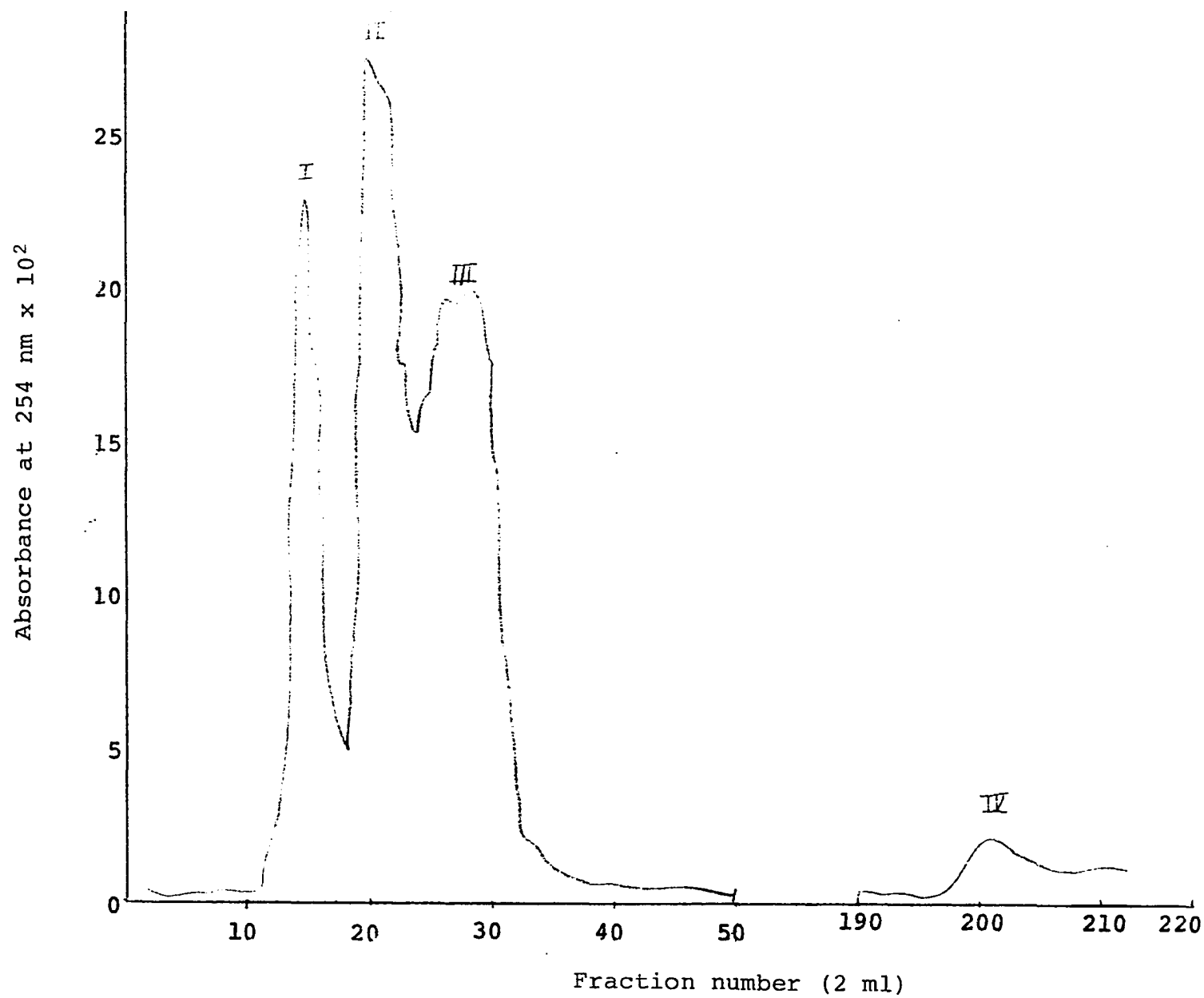
Figure 29. Elution of bacitracin fractions from
carboxymethyl cellulose column.
Absorbance measurements made at 255 nm.

I \equiv desamido bacitracin

II \equiv bacitracin F

III \equiv bacitracin A + B

IV \equiv minor component of commercial bacitracin



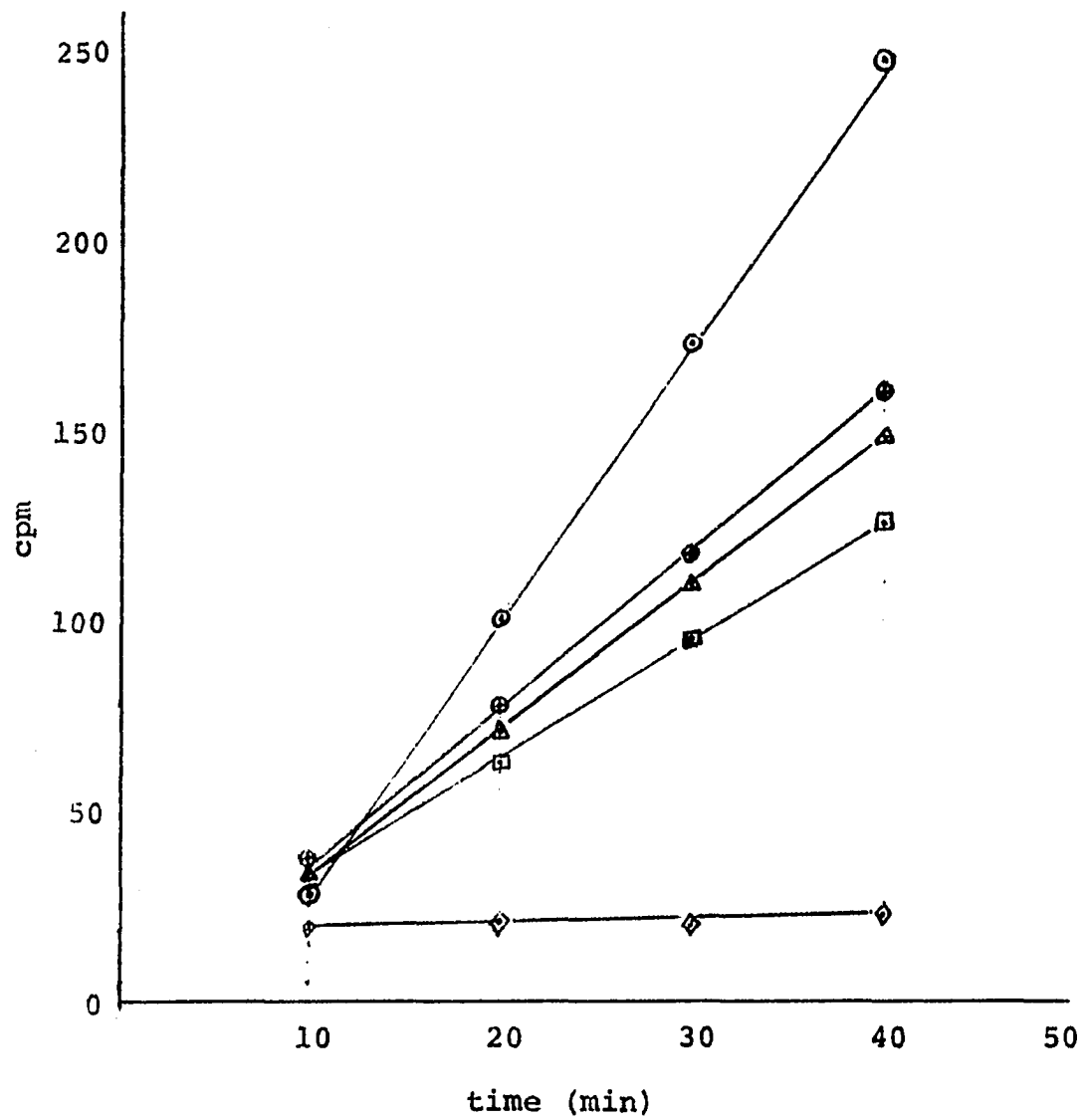


Figure 30. Assay of the bacitracin fractions as inhibitors of dextransucrase at ~50 mM.

- = control
- ⊕ = peak I
- △ = peak II
- = peak III
- ◇ = peak IV

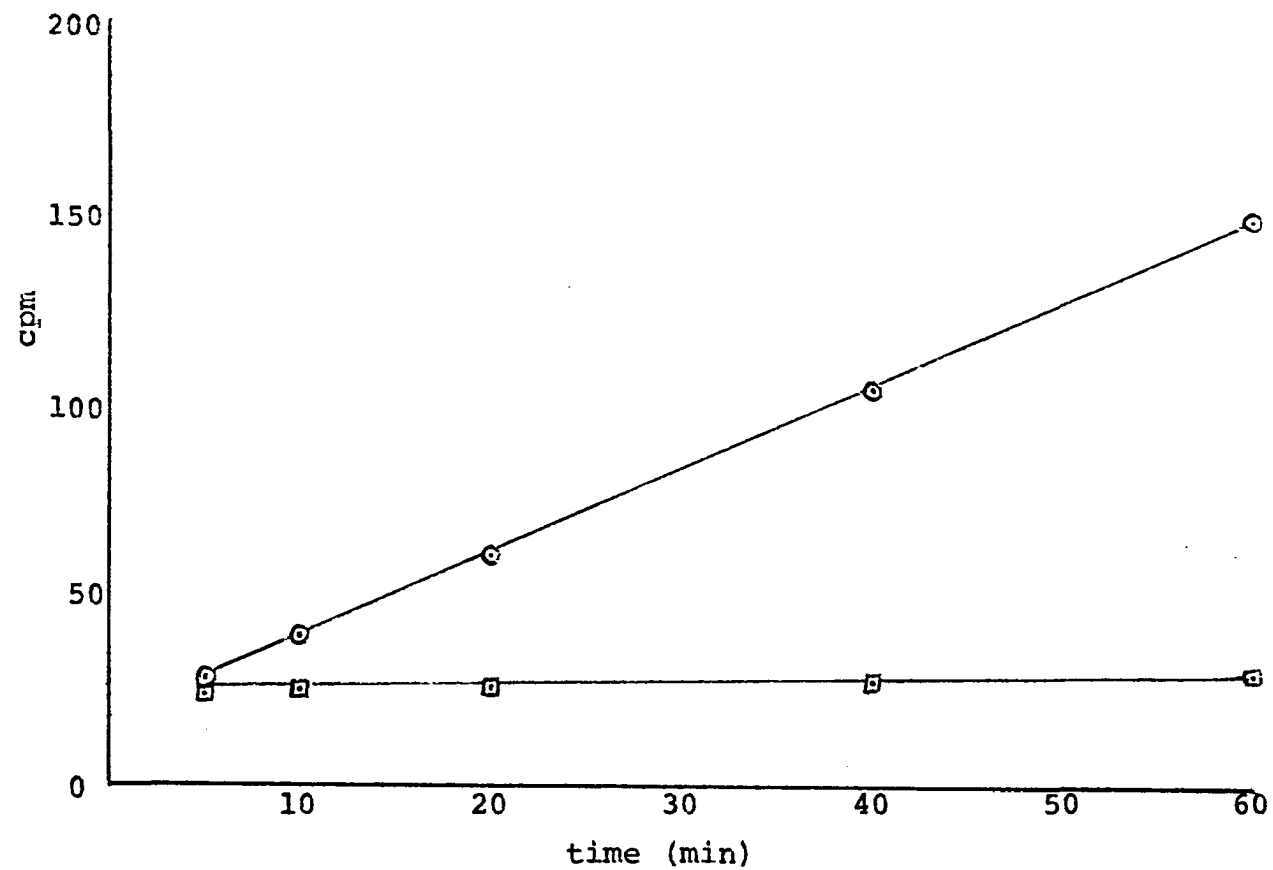


Figure 31. Assay of dextransucrase containing 5 mM zinc chloride.

○ = control

△ = zinc chloride

%I = 100

xylose acting as the acceptor as depicted in Figure 32. This reaction was carried out several times; however, the amount of material collected each time was quite small and the purification procedure did not lead to significantly large amounts of xylsucrose. A sample of xylsucrose was tested as an inhibitor of dextransucrase with results shown in Figure 33.

Due to the low yield of xylsucrose obtained, it was decided to undertake another approach to the preparation of substrate analogue inhibitors. In a paper by Genghof and Hehre (14), their studies with α -D-glucopyranosylfluoride as an alternative substrate for dextransucrase were described. This led us to the synthesis and testing of α -D-xylopyranosyl fluoride as a potential substrate analogue inhibitor of dextransucrase. The chemical synthesis of this compound resulted in the formation of large enough amounts to allow for a simplified purification which could then be assayed with dextransucrase. The initial work resulted in some interesting and encouraging observations. When α -D-xylopyranosyl fluoride was incubated with dextransucrase and then sucrose was added to the mixture a substantial amount of inhibition of the enzymatic activity was observed as shown in Figure 34.

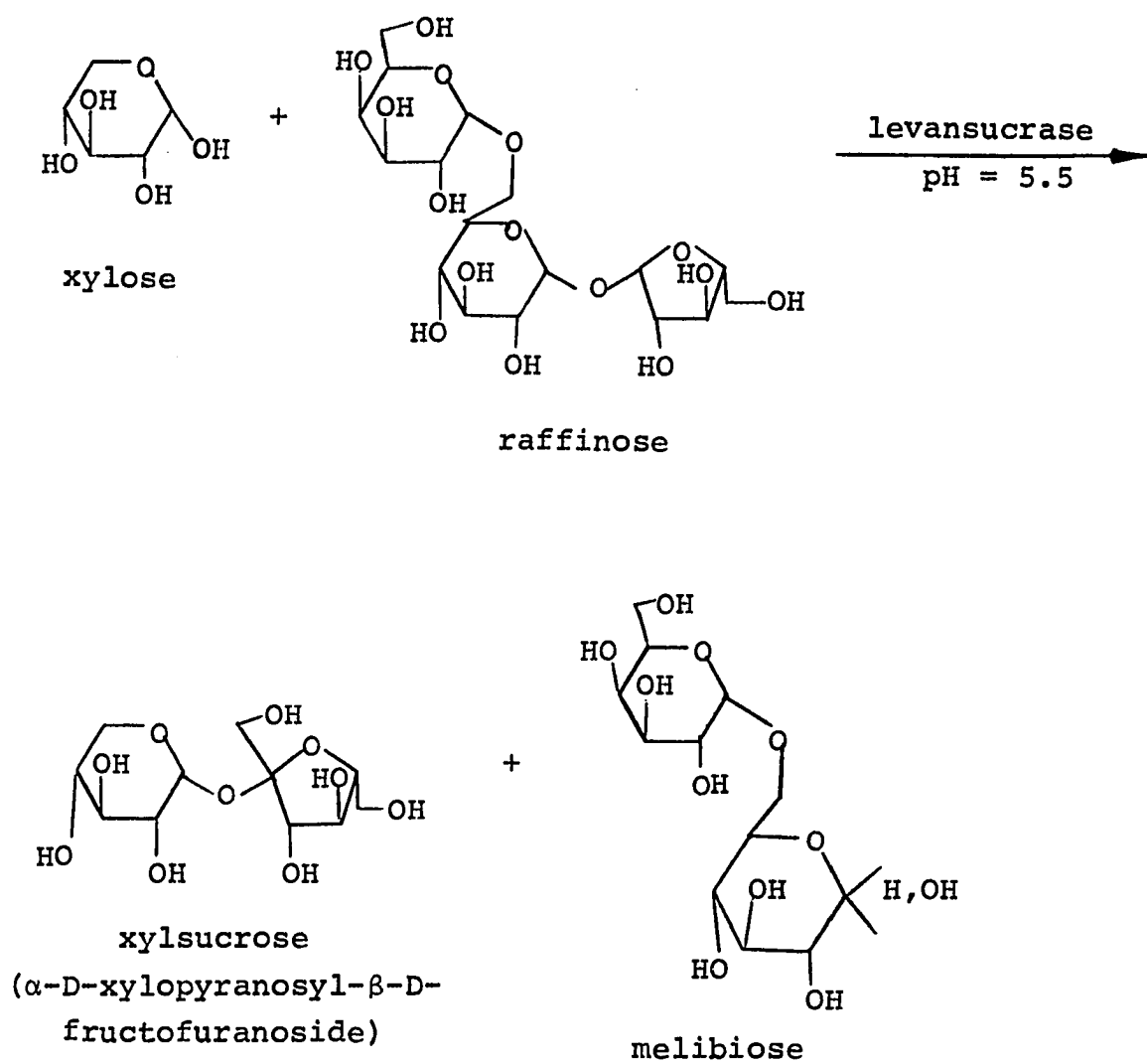


Figure 32. Enzymatic synthesis of xylsucrose.

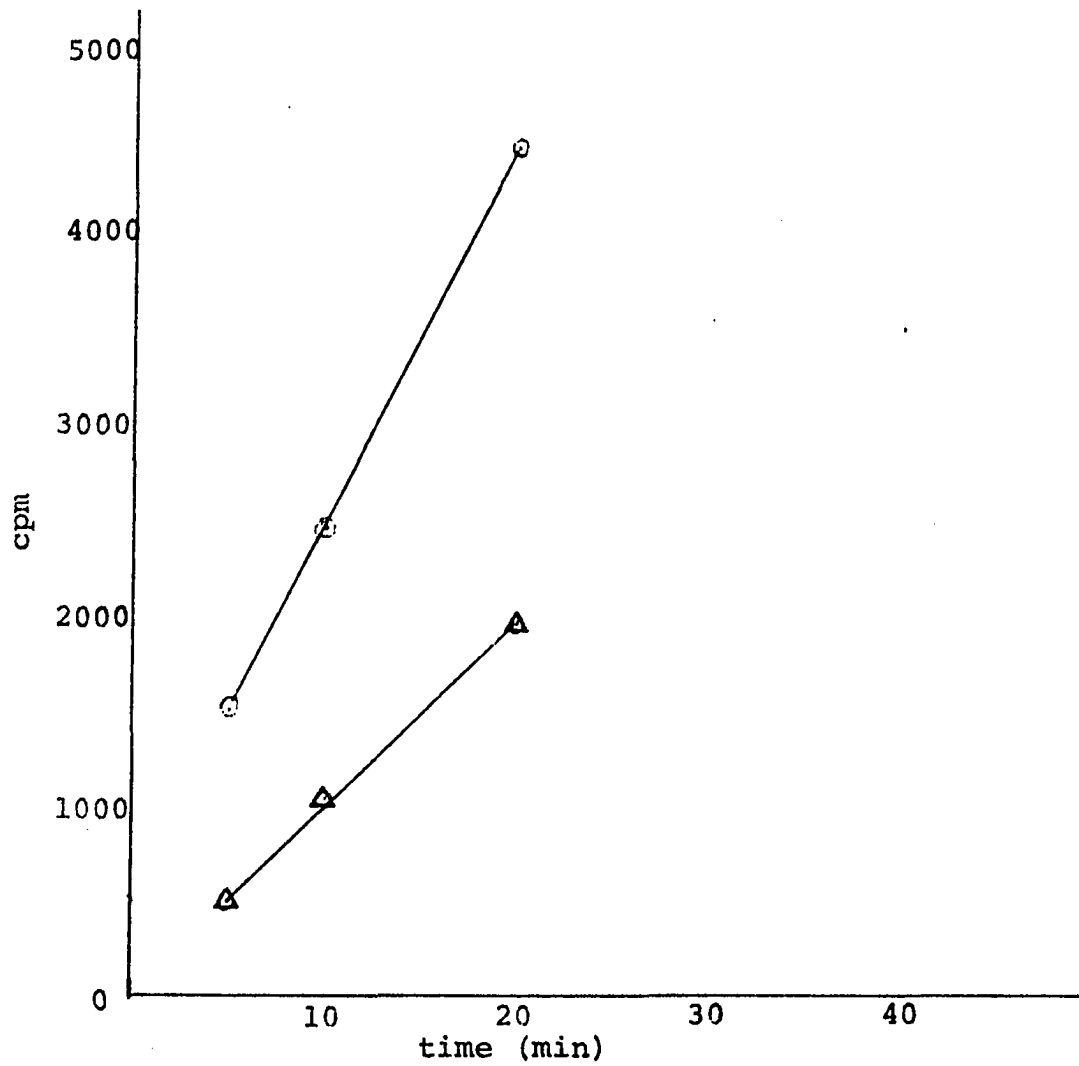


Figure 33. Assay of crude xylsucrose as an inhibitor of dextransucrase. Estimated concentration (upper limit) of xylsucrose is 43.2 mM.

○ = control

△ = xylsucrose

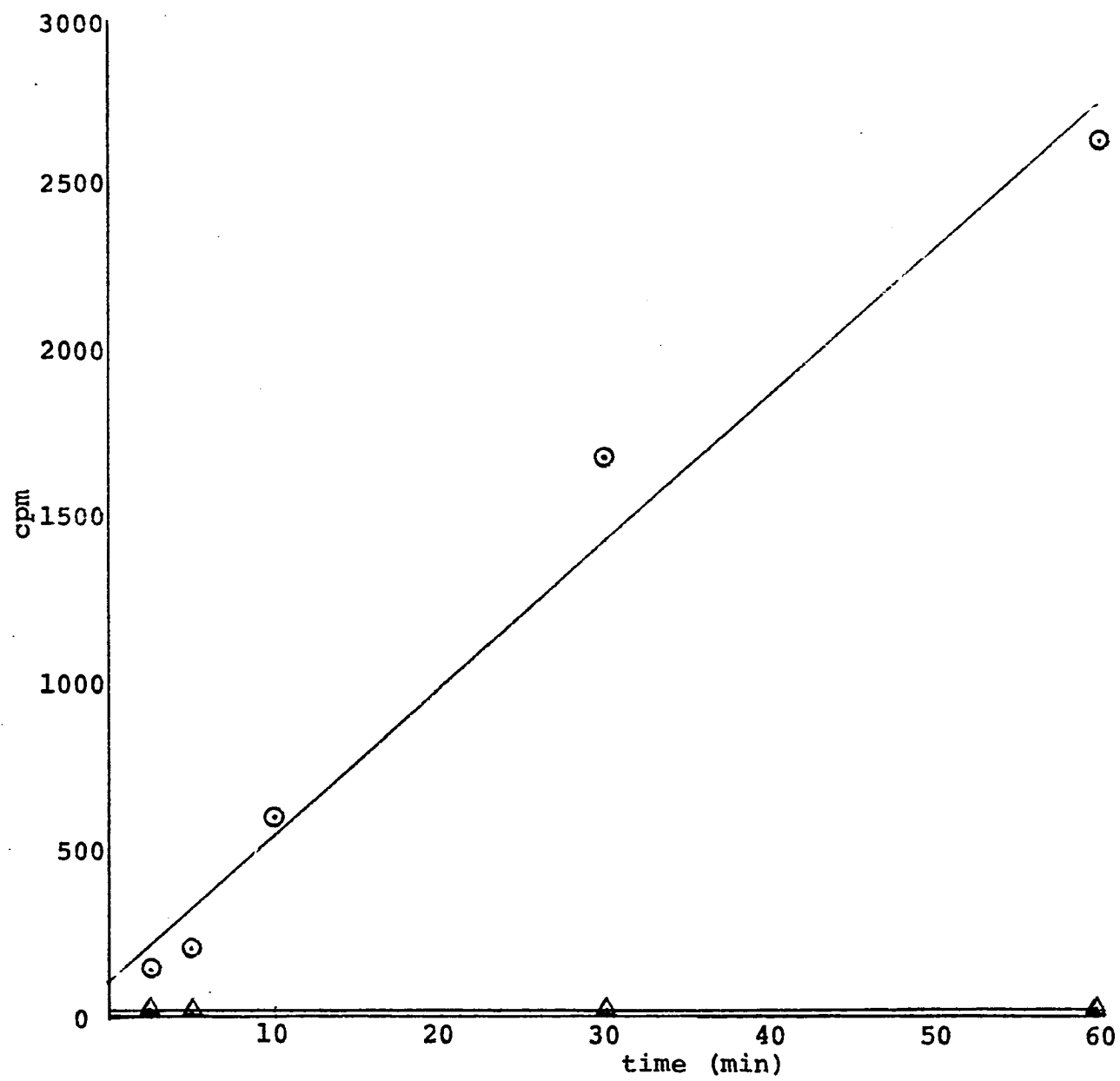
%I = 50.4

Figure 34. Assay of dextransucrase with and without 50 mM xylosyl fluoride.

⊙ = control

Δ = α-D-xylosyl fluoride

%I = 100



This assay was repeated again from the same batch of xylosyl fluoride and the results are shown in Figure 35.

The effects of α -D-xylose and of fluoride ion were ascertained by incubating the dextransucrase enzyme with a mixture consisting of buffer at pH 5, 70 mM xylose and 70 mM sodium fluoride. The results of this assay are shown in Figure 36 indicating that there was no effect on the enzymatic activity by these two substances.

Several preparations of α -D-xylopyranosyl fluoride were carried out in efforts to obtain higher yields and more highly purified material. The results of the assays of some of these preparations with dextransucrase are shown in Figure 37 and Table II.

The variability of the inhibition can be attributed to the amount of α -D-xylopyranosyl fluoride present in the preparation. This substance is labile towards acid hydrolysis and the hydrolysis is probably somewhat autocatalytic since one of the products of the hydrolysis is hydrofluoric acid.

Further studies on the mechanism of action centered around the use of sucrose analogues derived from sucrose. Substitution of a fluorine atom for a hydroxyl group was chosen as a goal due to the size of the covalently linked fluorine atom (1.35 Å) versus the size of a

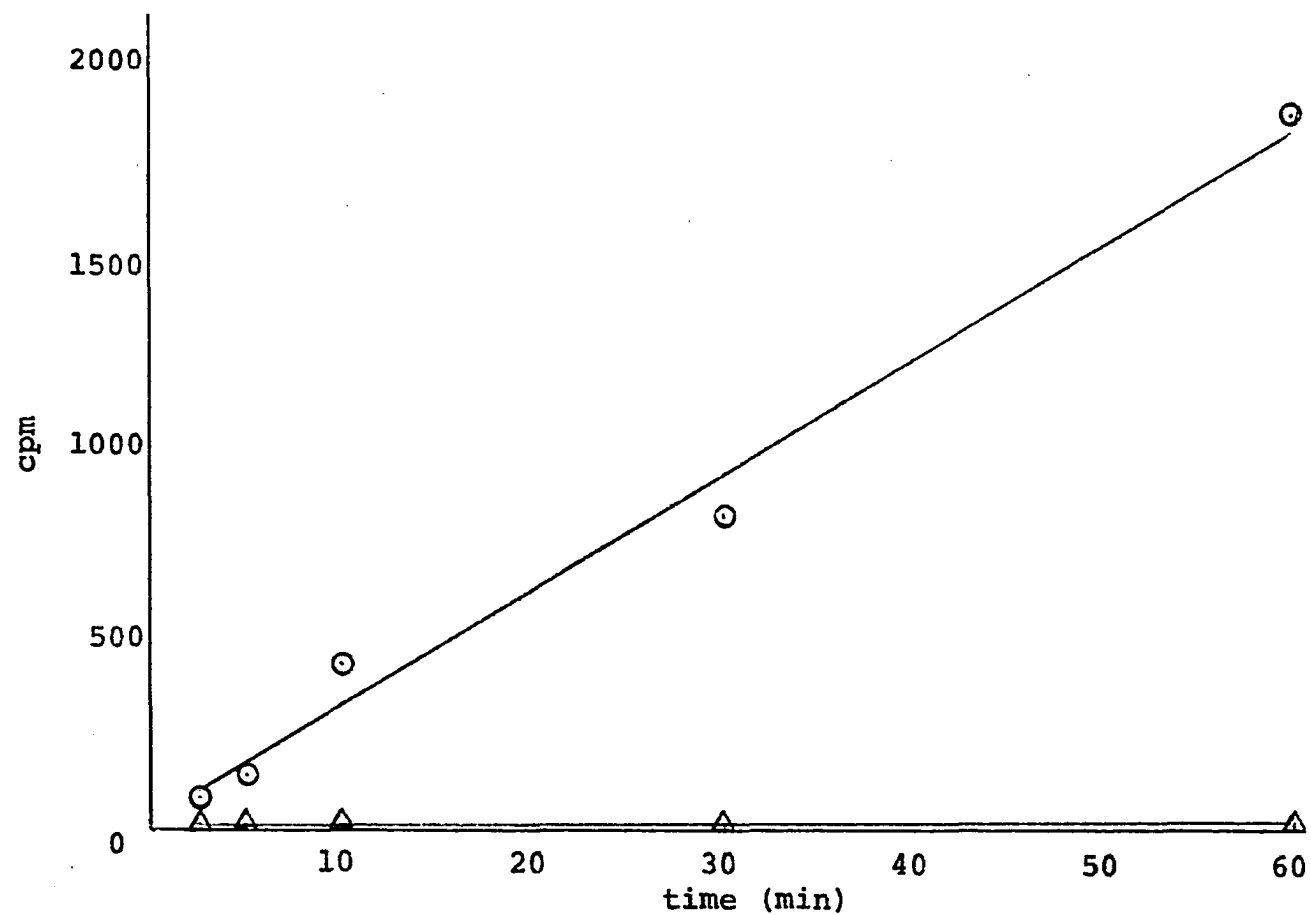


Figure 35. Assay of dextransucrase using α -D-xylosyl fluoride at ~50 mM as an inhibitor.

○ = control

△ = α -D-xylosyl fluoride

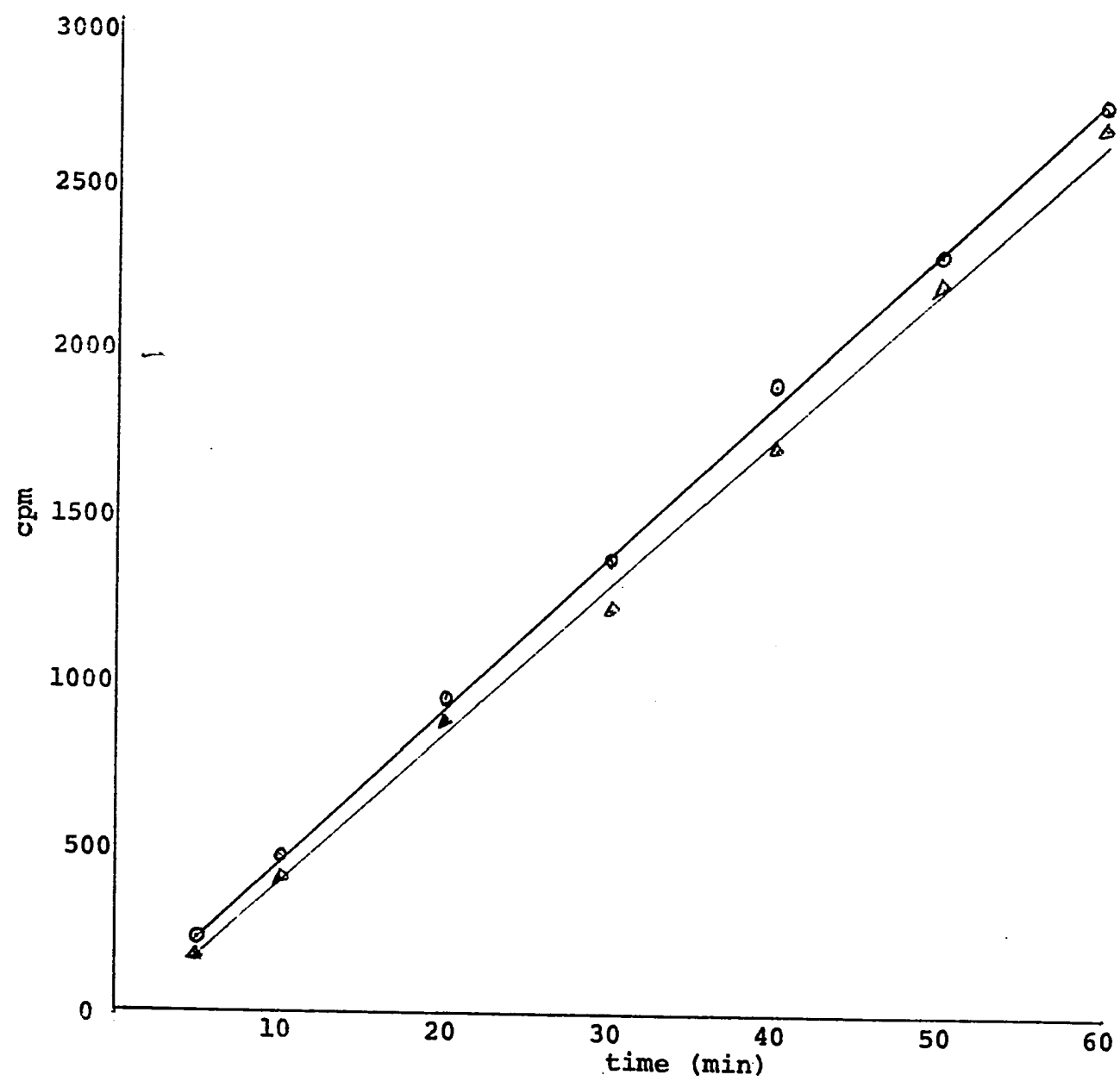
%I = 100

Figure 36. Assay of dextransucrase with 70 mM
sodium fluoride and 70 mM xylose.

○ = control

△ = NaF + xylose

%I = 3



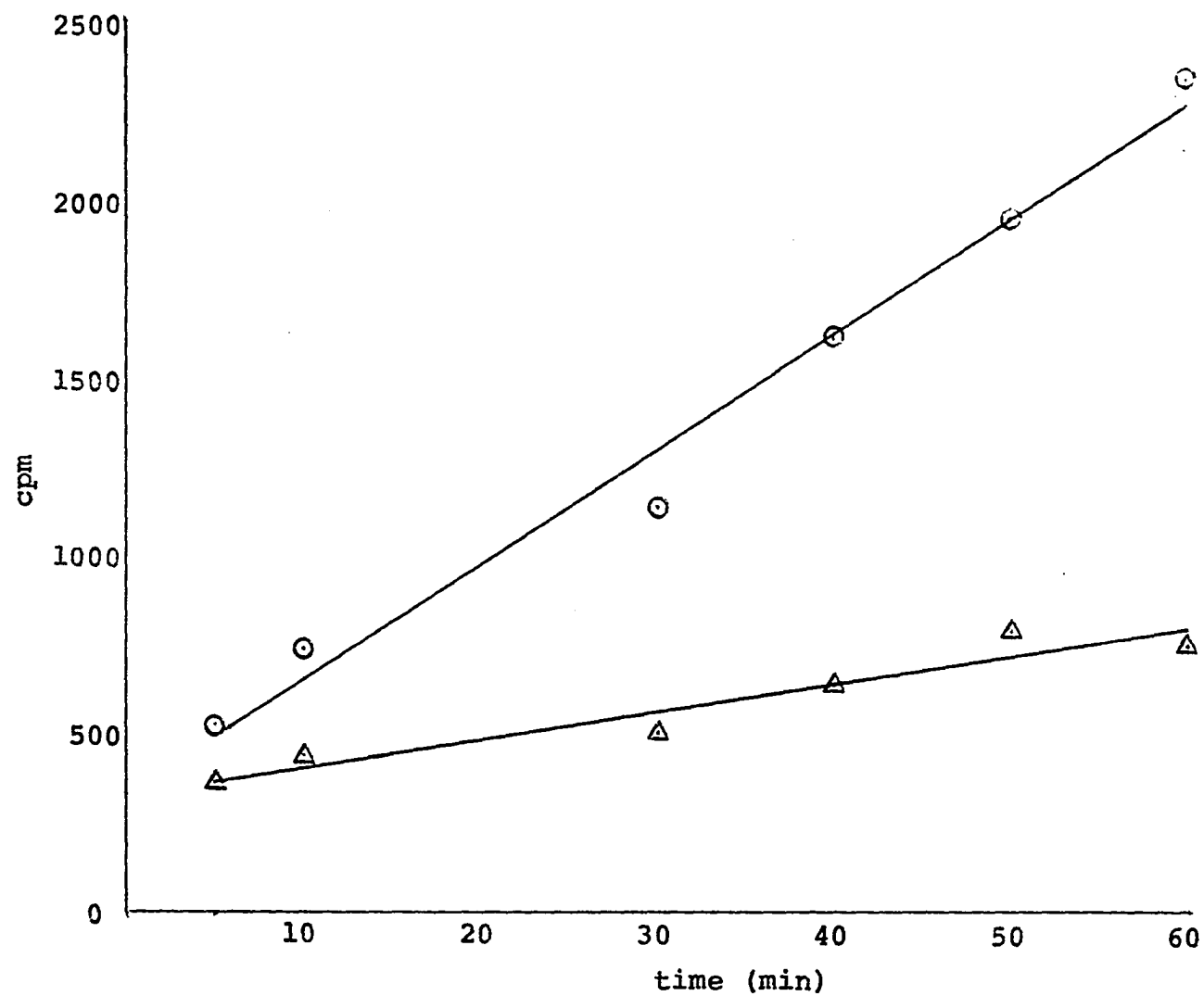


Figure 37. Assay of dextranucrase with ~50 mM xylosyl fluoride.

○ = control Δ = xylosyl fluoride %I = 76.6

Table II. Results of assays of different preparations of α -D-xylopyranosyl fluoride.

Preparation	% I ^a
1	41.9
2	32.5
3	33.9
4	71.8
5	83.3
6	80.6
7	70.4
8	76.6

^aThe percent of inhibition was obtained by finding the least squares slope of the control reaction and of the inhibited reaction and then comparing this data as described in the equation;

$$\% I = \frac{\text{slope of control rx} - \text{slope of inhibited rx}}{\text{slope of control rx}} \times 100\%$$

hydroxyl group ($1.40 \overset{\text{O}}{\text{\AA}}$) and its ability to form hydrogen bonds by acting as a hydrogen bond acceptor (39). Towards this goal the synthesis of 6,1',6'-trideoxy-6,1',6'-tri-fluorosucrose was attempted by Sn_2 reactions carried out on various sulfonate derivatives of sucrose using mostly potassium fluoride as a source of fluoride ions. This method was tried with several variations. The product mixture was assayed for inhibition towards dextranucrase. Examples of the results of these assays are shown in Figures 38 through 41.

Similar results were obtained using acetate esters instead of benzoate esters, tosyl derivatives of sucrose instead of tripsyl derivatives and hexamethylphosphoramide (HMPA) as a solvent instead of N,N,-dimethylformamide (DMF). One exception was observed : when lithium fluoride was substituted for potassium fluoride in the reaction sequence, only 56% inhibition was observed with the final product. However, when the products of the reaction with lithium fluoride were refluxed with potassium fluoride in N,N-dimethylformamide inhibition of the resultant products towards the activity of dextranucrase of 100% was observed again as shown in Figure 42. This may be a direct result of the availability of free fluoride ion in that potassium fluoride is more ionic in character than lithium fluoride

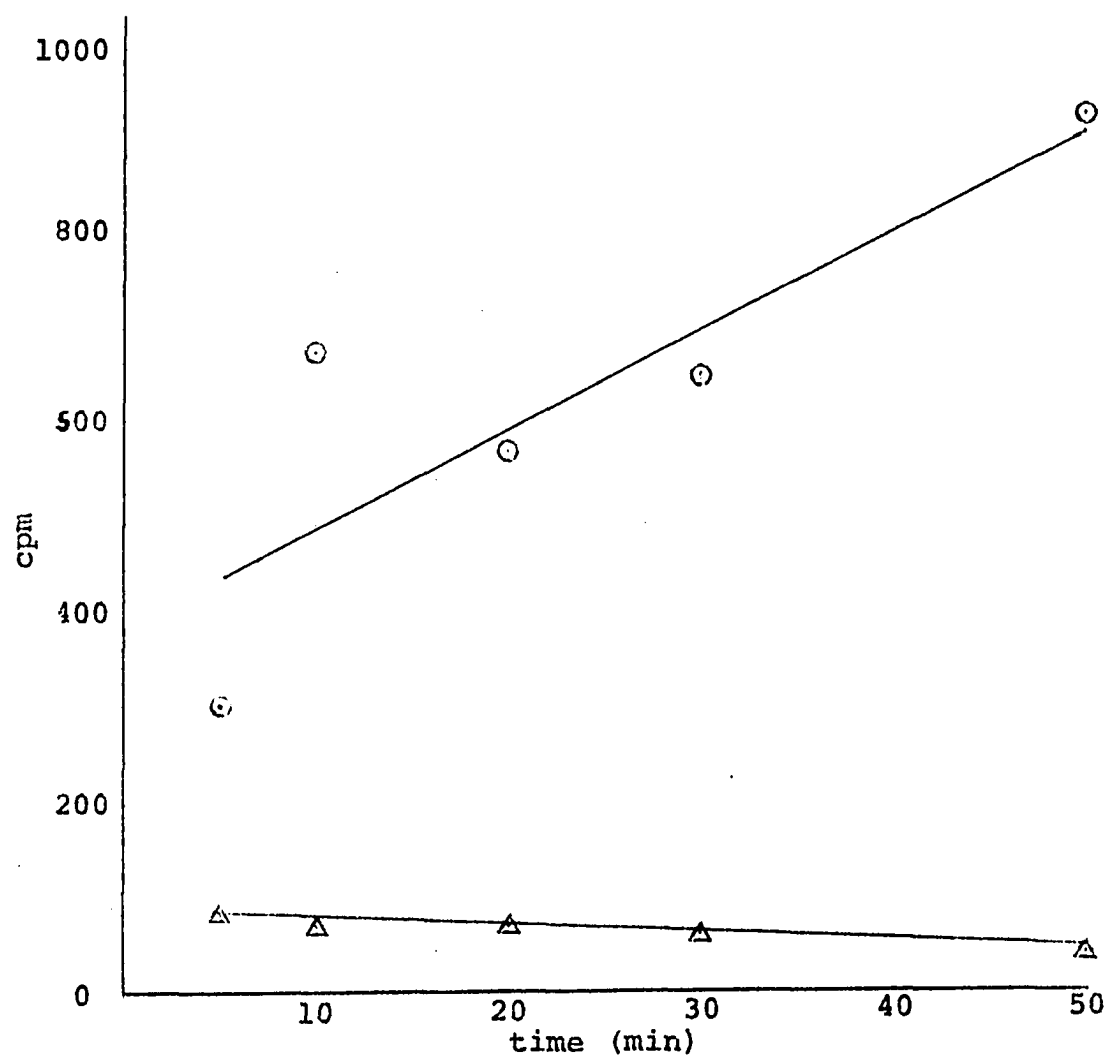


Figure 38. Assay of dextranucrase with the mixture of inhibitors derived from tri-O-tripsylsucrose.

○ = control

△ = inhibitor

%I = 100

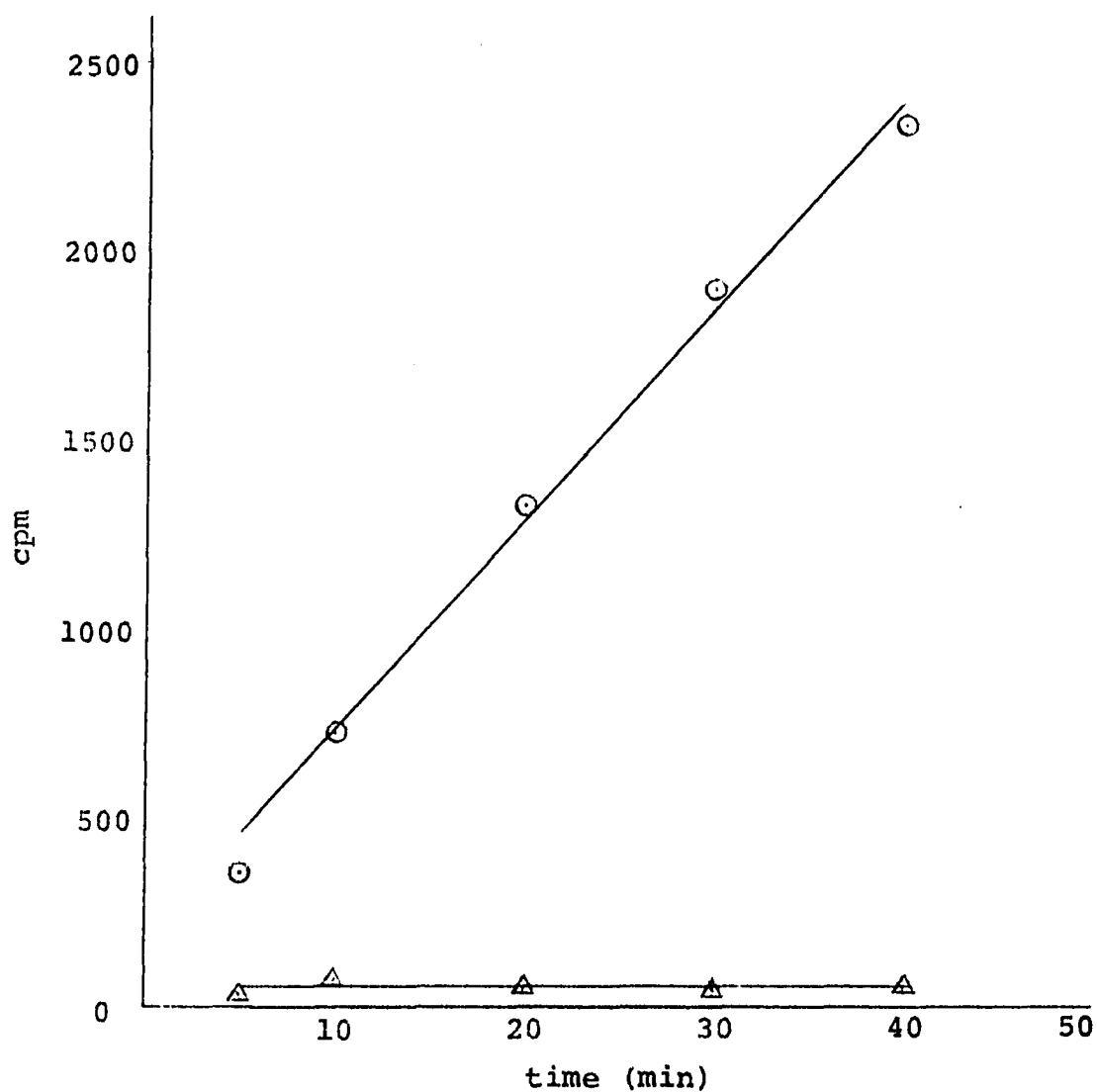


Figure 39. Assay of dextranucrase with the mixture of inhibitors derived from tri-O-tripsylsucrose.

○ = control

△ = inhibitor

%I = 100

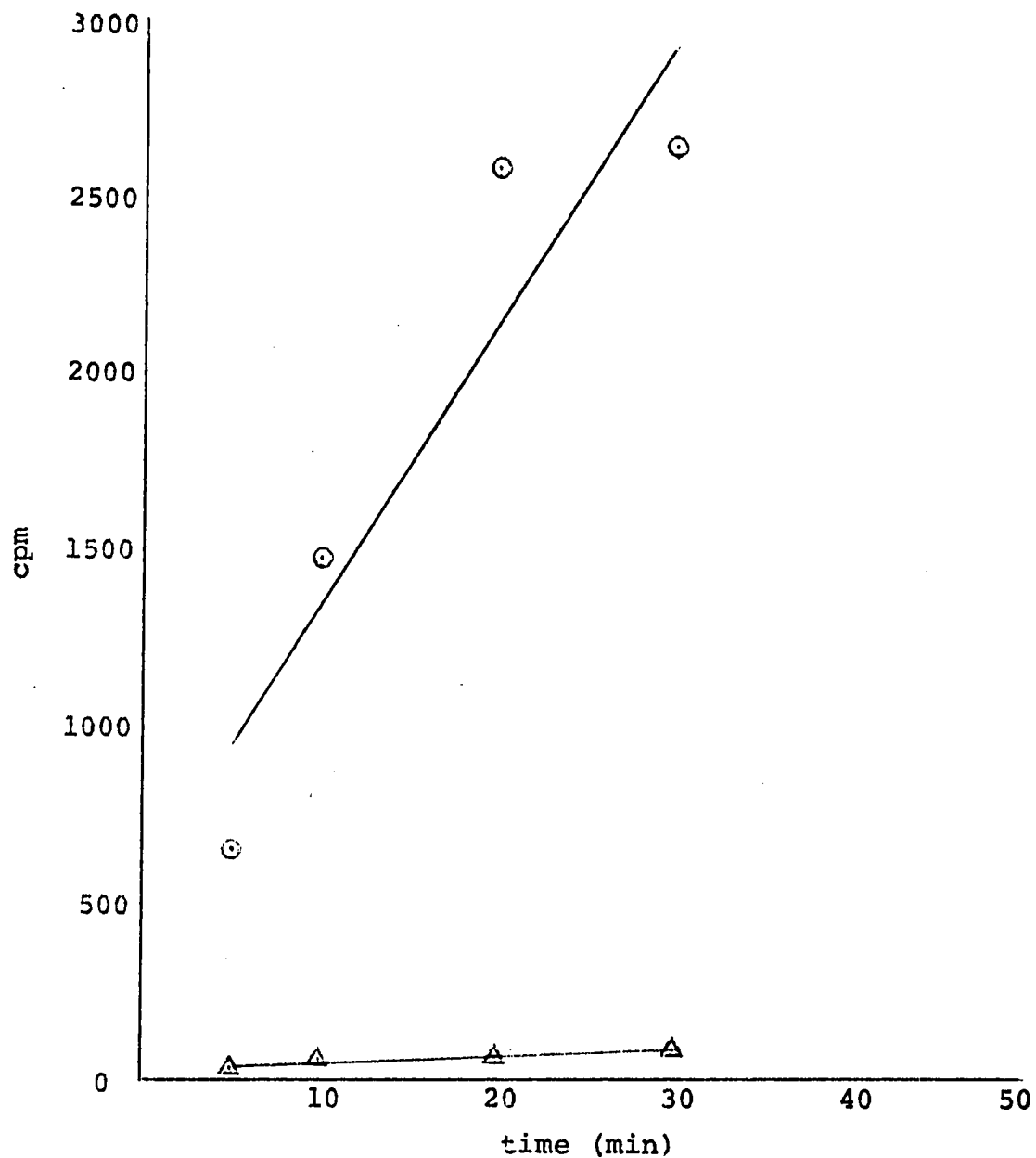


Figure 40. Assay of dextranucrase with the mixture of products derived from tri-O-tripsylsucrose.

○ = control

△ = inhibitor

%I = 98

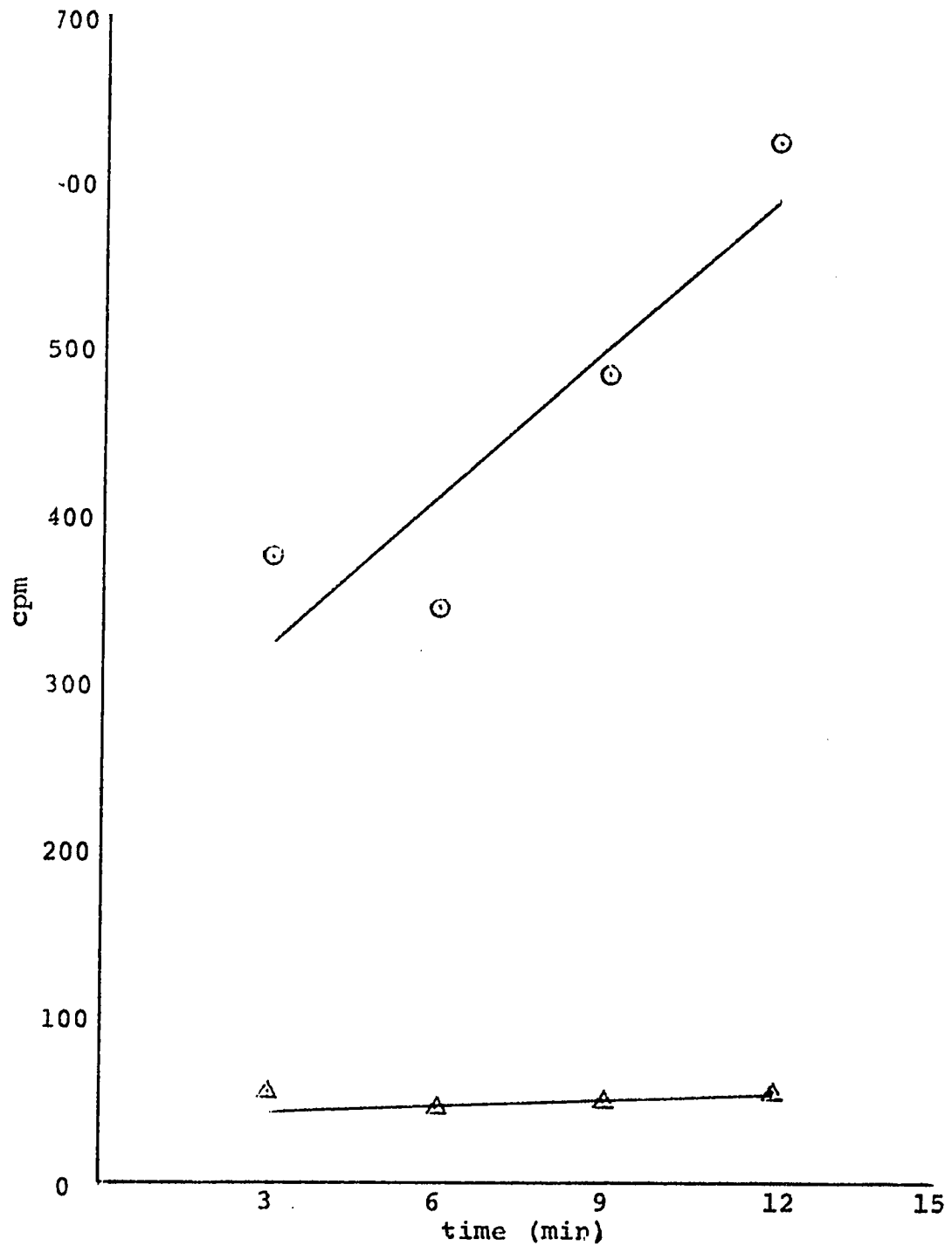


Figure 41. Assay of dextranucrase with the mixture derived from tri-O-tripsylsucrose.

○ = control

△ = inhibitor.

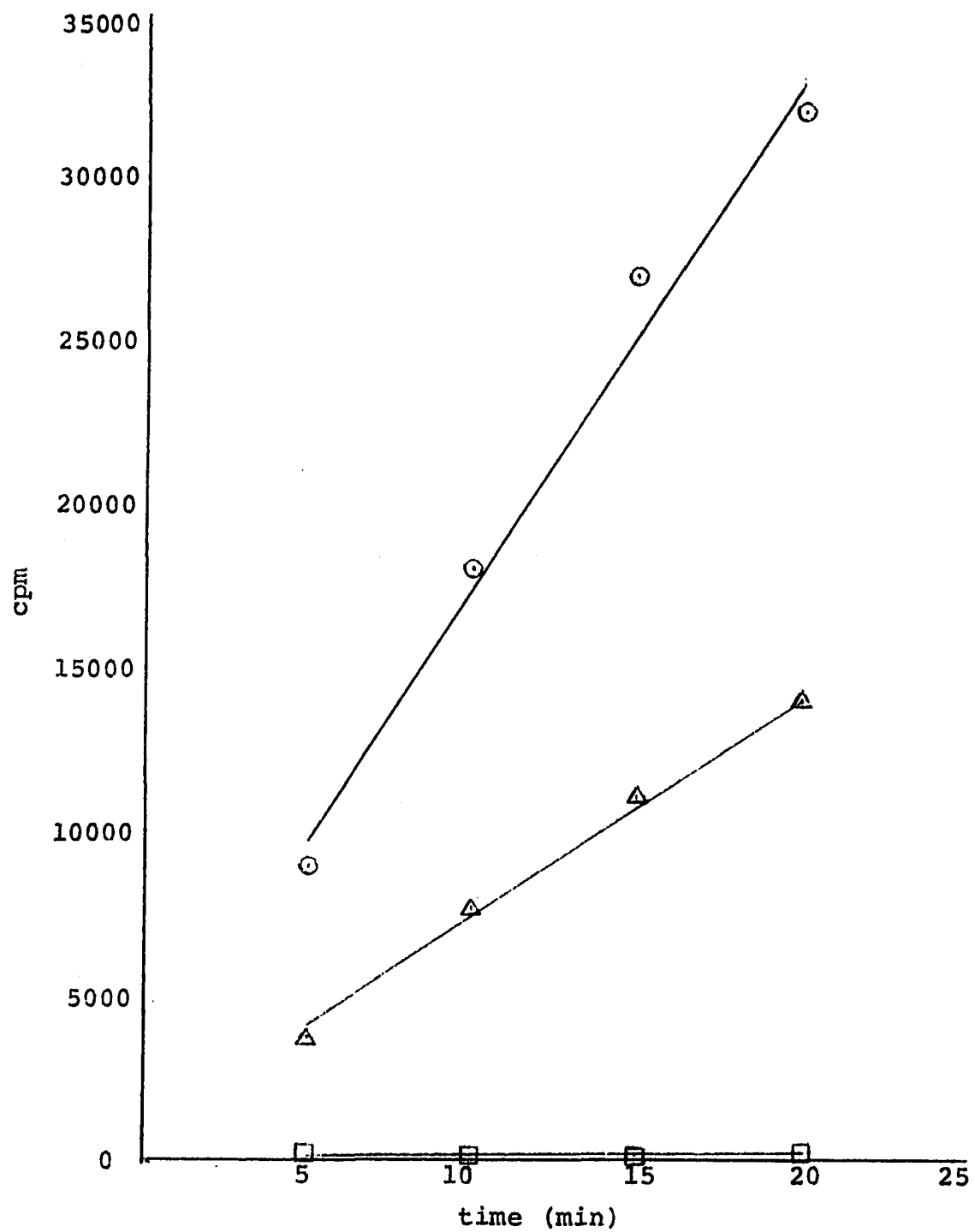
%I = 95

Figure 42. Assay of dextransucrase with the mixture of products derived from tri-O-tripsyl-sucrose pentabenzoate treated first with lithium fluoride as a fluorinating agent followed by potassium fluoride as a fluorinating agent.

○ = control

△ = lithium fluoride %I = 56.4

□ = potassium fluoride %I = 100



and consequently there is a higher concentration of fluoride ion per mole of dissolved potassium fluoride than there is per mole of dissolved lithium fluoride. All of the reaction conditions utilized in the sulfonate displacement reactions with fluoride ion resulted in the formation of a complex mixture of products.

In one typical case, paper chromatography showed the presence of fourteen components after deacylation, i.e., the final step in the reaction. Since the final material in all these cases was a mixture which was not easily resolvable, several other synthetic routes were taken under consideration. One of these was made very appealing by the commercial availability of a very mild fluorinating reagent, namely diethylaminosulfur trifluoride (DAST). The synthetic work described earlier resulted in the formation of an analogue of sucrose, mainly 6,6'-dideoxy-6,6'-difluoro-sucrose, structurally very similar to sucrose, which on incubation with dextransucrase proved to be an inhibitor as shown in Figures 43 and 44.

In addition to the synthesis of 6,6'-difluoro-6,6'-dideoxysucrose, the synthesis of the mixed 6-fluoro-6-deoxysucrose and 6'-fluoro-6'-deoxysucrose resulted in enough material to carry out two assays with dextransucrase. These assays are shown in Figures 45 and 46.

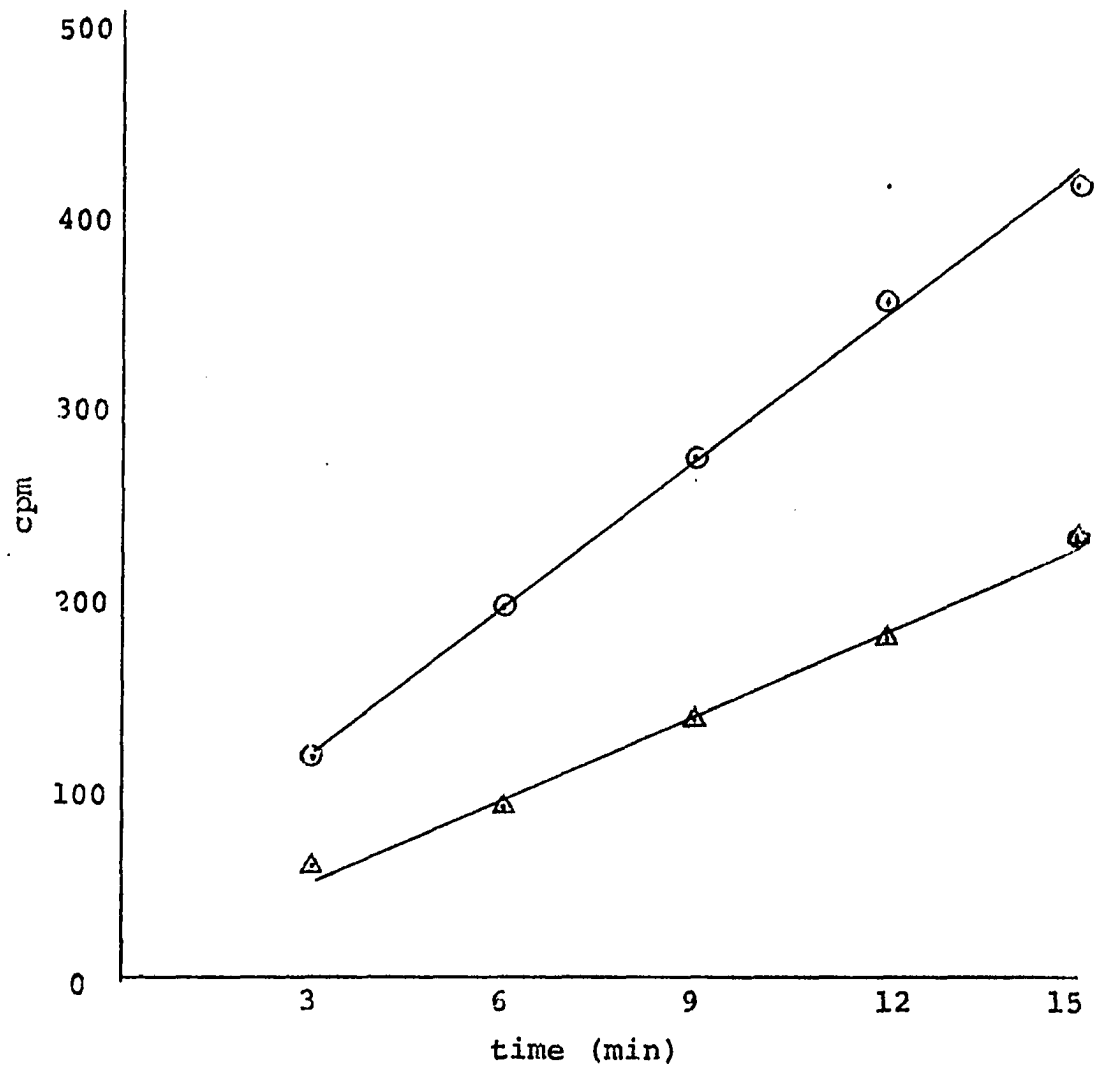


Figure 43. Assay of dextranucrase with and without 6,6'-difluoro-6,6'-dideoxysucrose at -150 mM.

○ = control

△ = inhibitor

%I = 43

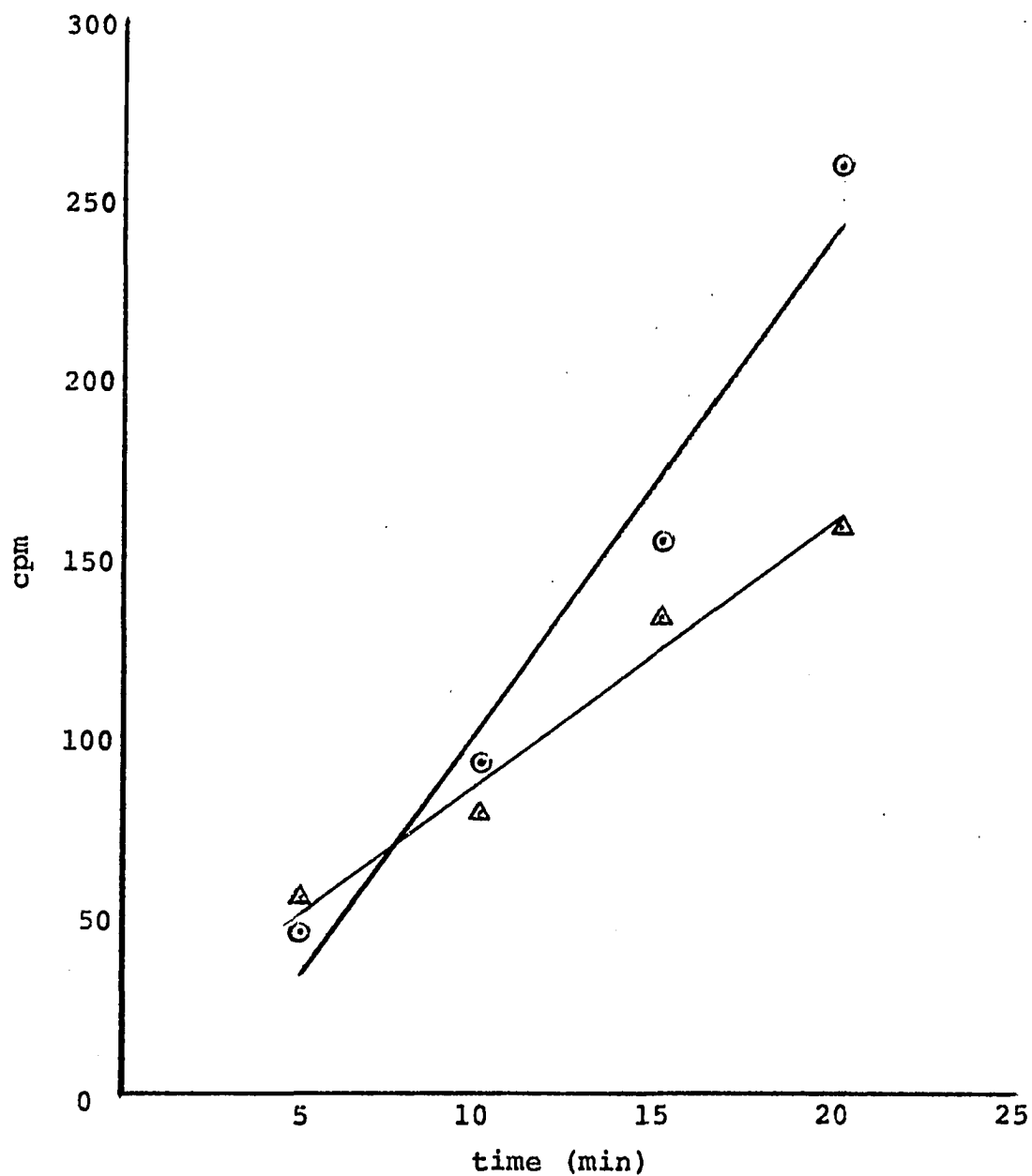


Figure 44. Assay of dextranucrase with 6,6'-difluoro-6,6'-dideoxysucrose at ~150 mM. Preincubation time is 120 minutes in this assay.

○ = control

△ = inhibitor

%I = 48

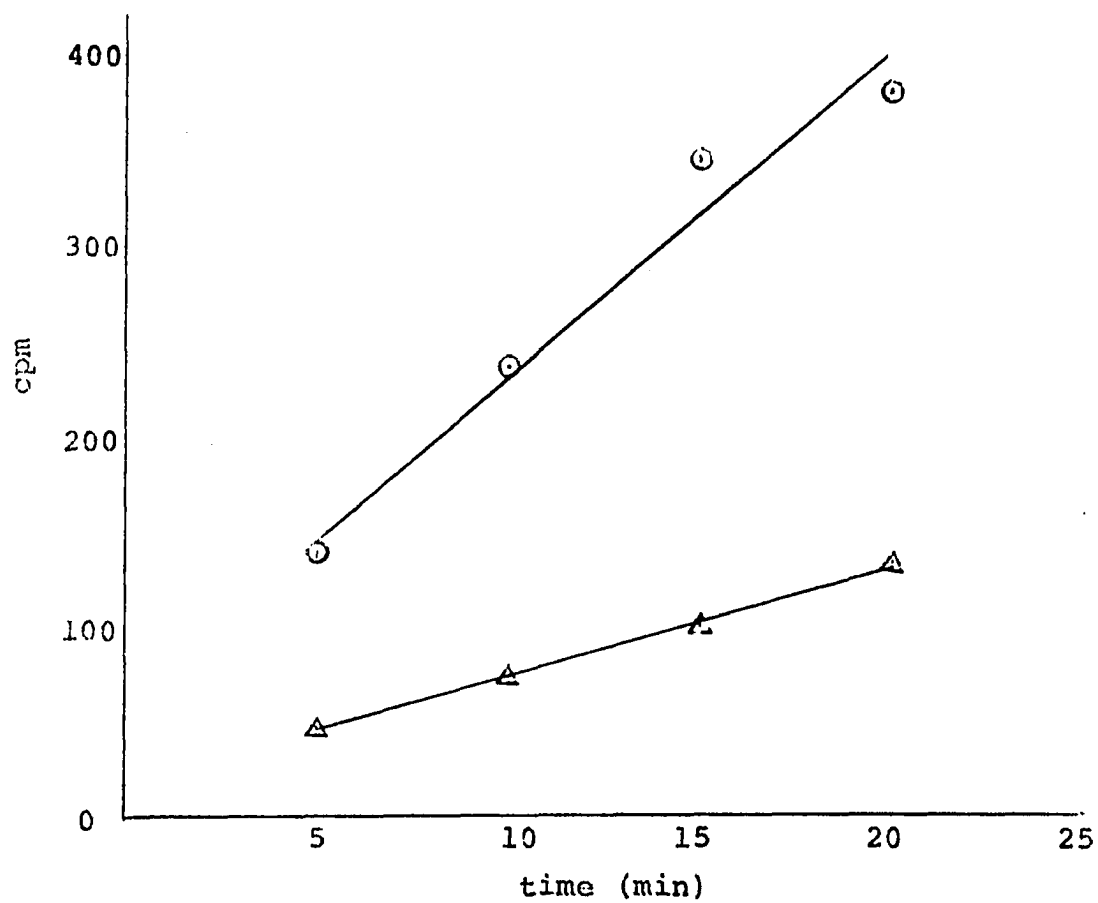


Figure 45. Assay of dextranucrase with a mixture of 6-fluoro-6-deoxysucrose and 6'-fluoro-6'-deoxysucrose at a combined concentration of ~150 mM.

○ = control

△ = inhibitor

%I = 67

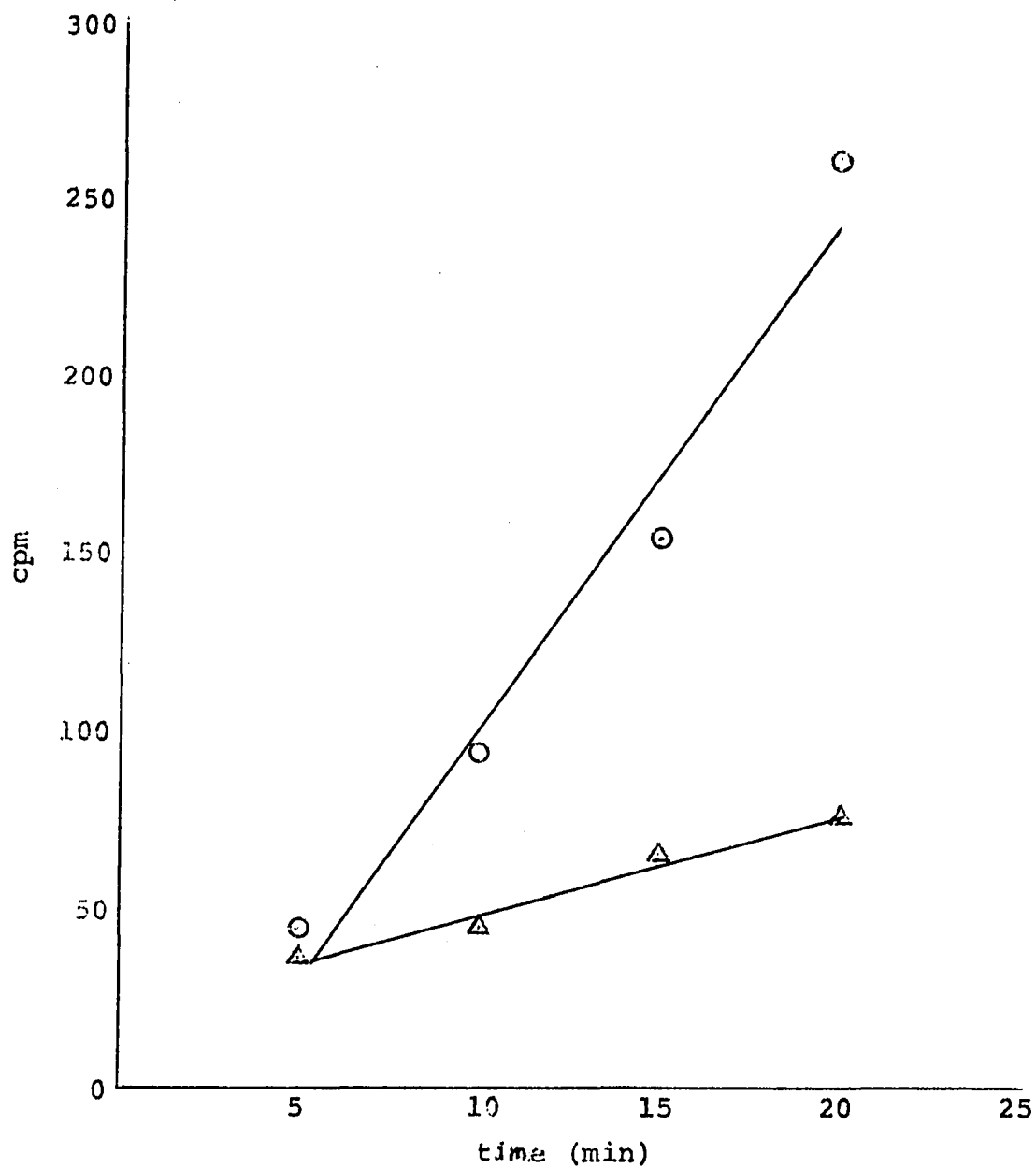


Figure 46. Assay of dextranucrase with a mixture of 6-fluoro-6-deoxysucrose and 6'-fluoro-6'-deoxysucrose at a combined concentration of ~150 mM. Preincubation time in this assay was for 120 minutes.

○ = control

△ = inhibitor

%I = 80

The results of the work with the dibromo and dichloro analogues of sucrose, i.e., 6,6'-dibromo-6,6'-dideoxysucrose and 6,6'-dichloro-6,6'-dideoxysucrose are shown in Figures 47 through 50.

Comparing the results obtained from the assays of dextranucrase with the various inhibitors brings forth the observation that the most potent inhibitors were those resulting from the action of potassium fluoride upon sucrose-sulfonates, dibromosucrose and α -D-xylopyranosyl fluoride.

The following is submitted as a hypothesis for the reasons behind the observed results.

Beginning with α -D-xylopyranosyl fluoride it has been observed by us that this material is relatively labile and will undergo hydrolysis upon standing at room temperature over a period of one to two months. In addition, the structural analogue α -D-glucopyranosyl fluoride which is a well-known substrate for dextranucrase at higher concentrations exhibits inhibitory activity towards the enzyme. Based upon these facts and the fact that α -D-xylopyranosyl fluoride is an inhibitor of this enzyme there is the possibility that α -D-xylopyranosyl fluoride is acting as a glycosylating agent and in this manner reacting with dextranucrase at several points including but not limited to the binding site(s) and the catalytic site(s).

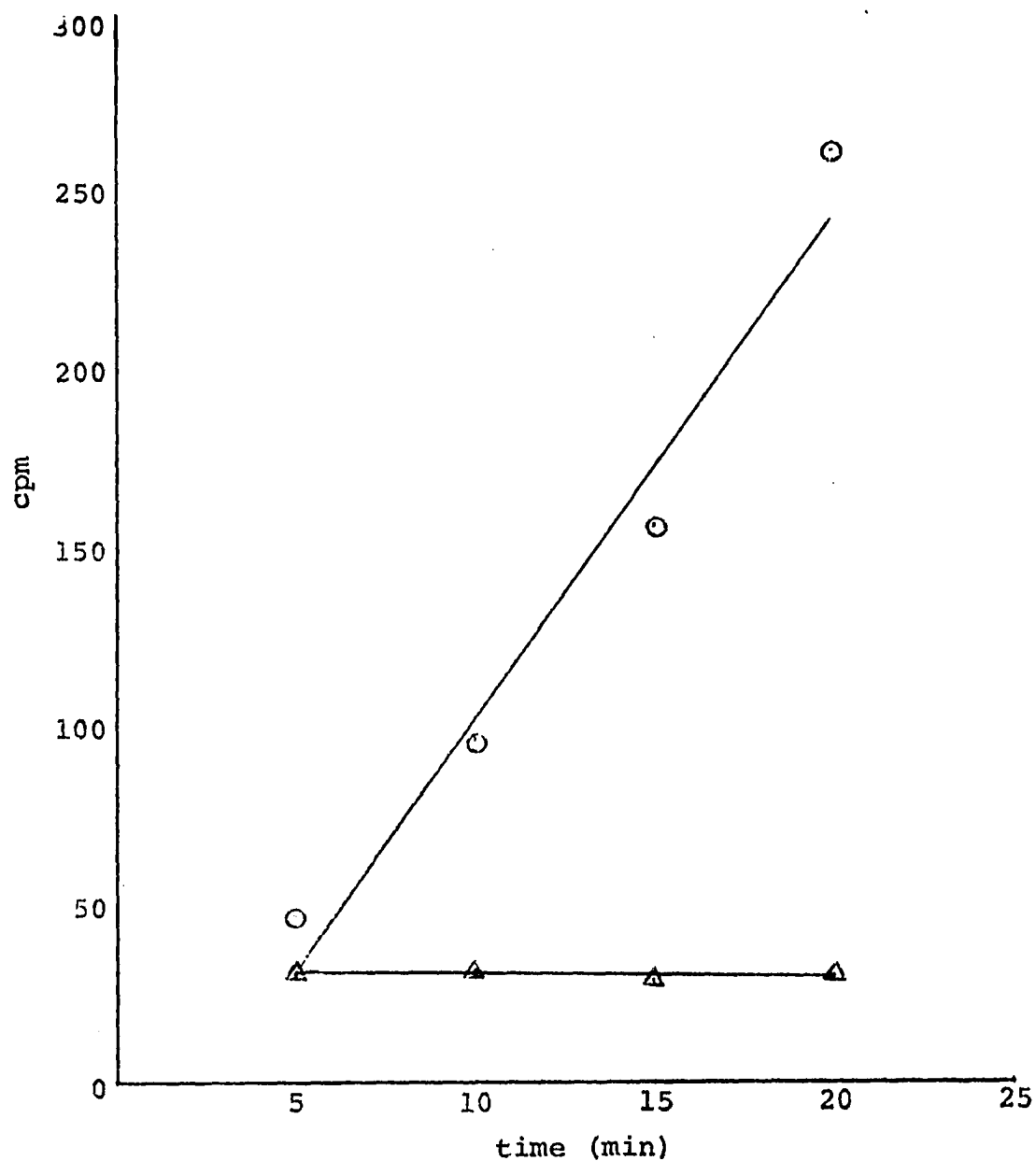


Figure 47. Assay of dextranucrase with 6,6'-dibromo-6,6'-dideoxysucrose at ~150 mM for 120 minutes pre-incubation.

○ = control

△ = inhibitor

%I = 100

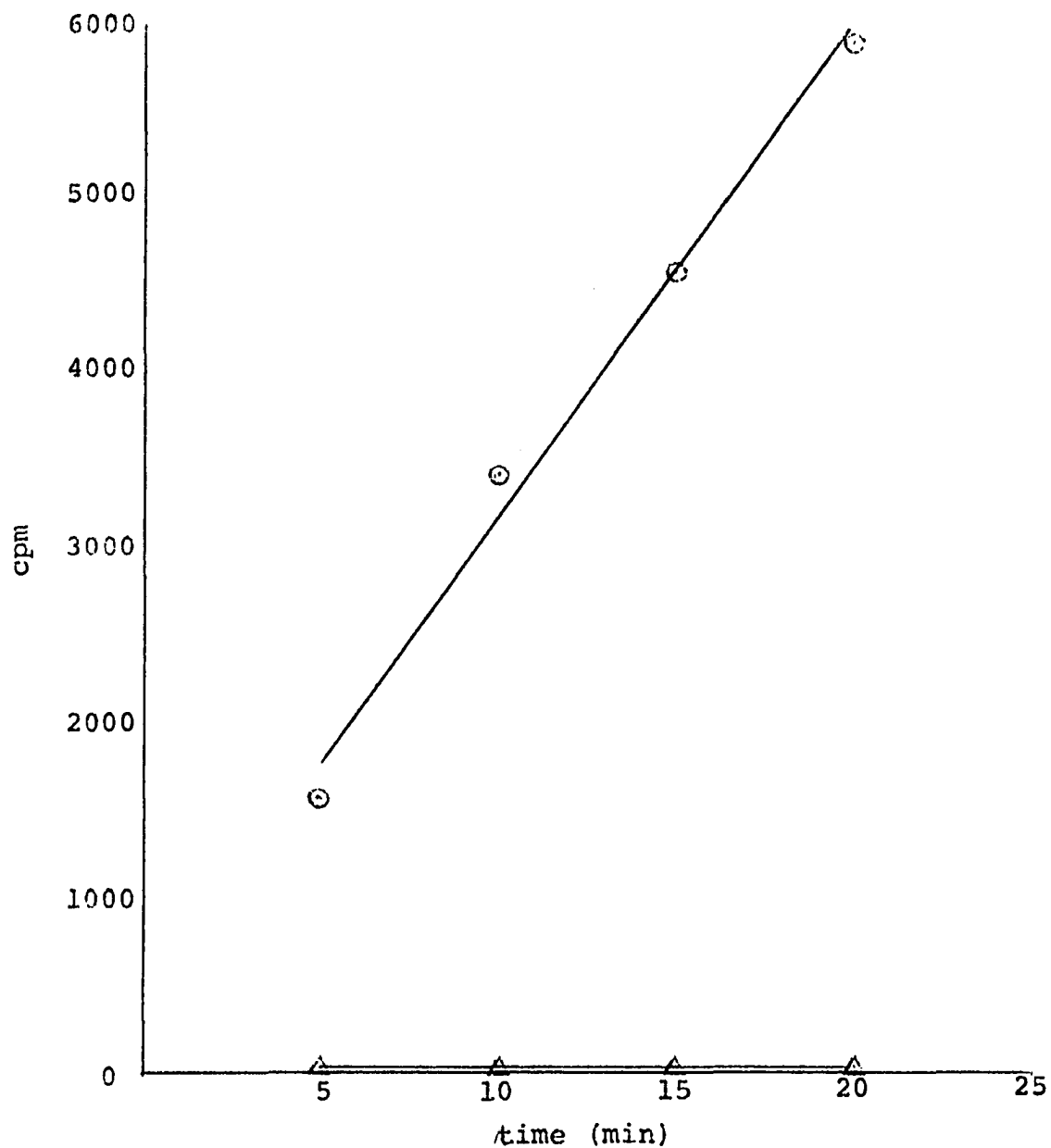


Figure 48. Assay of dextranucrase with 6,6'-dibromo-6,6'-dideoxysucrose at ~150 mM for a 10 minute pre-incubation.

○ = control

△ = inhibitor

%I = 100

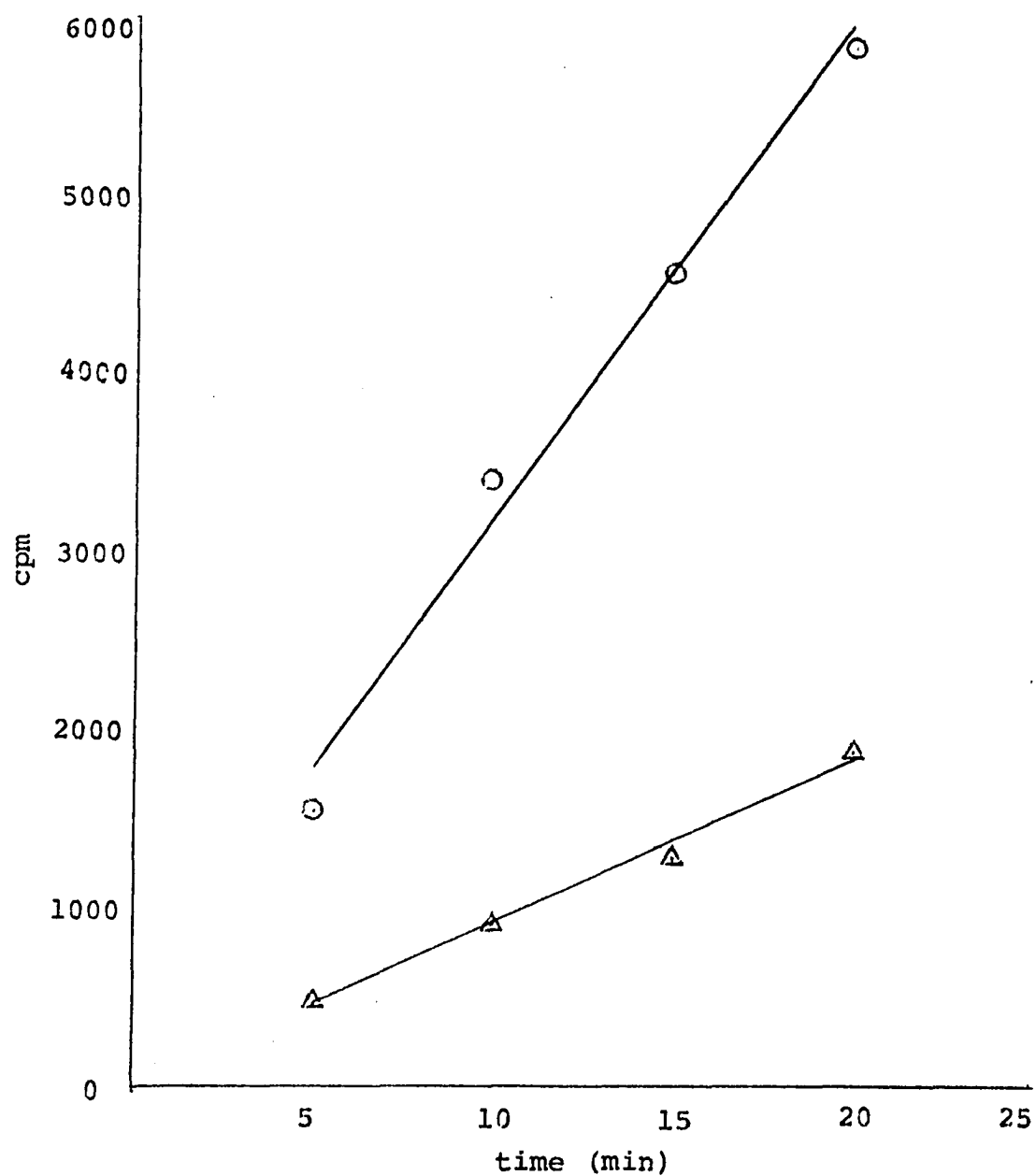


Figure 49. Assay of dextranucrase with 6,6'-dichloro-6,6'-dideoxysucrose (150 mM). Preincubation for 10 minutes. .

○ = control

△ = inhibitor

%I = 67

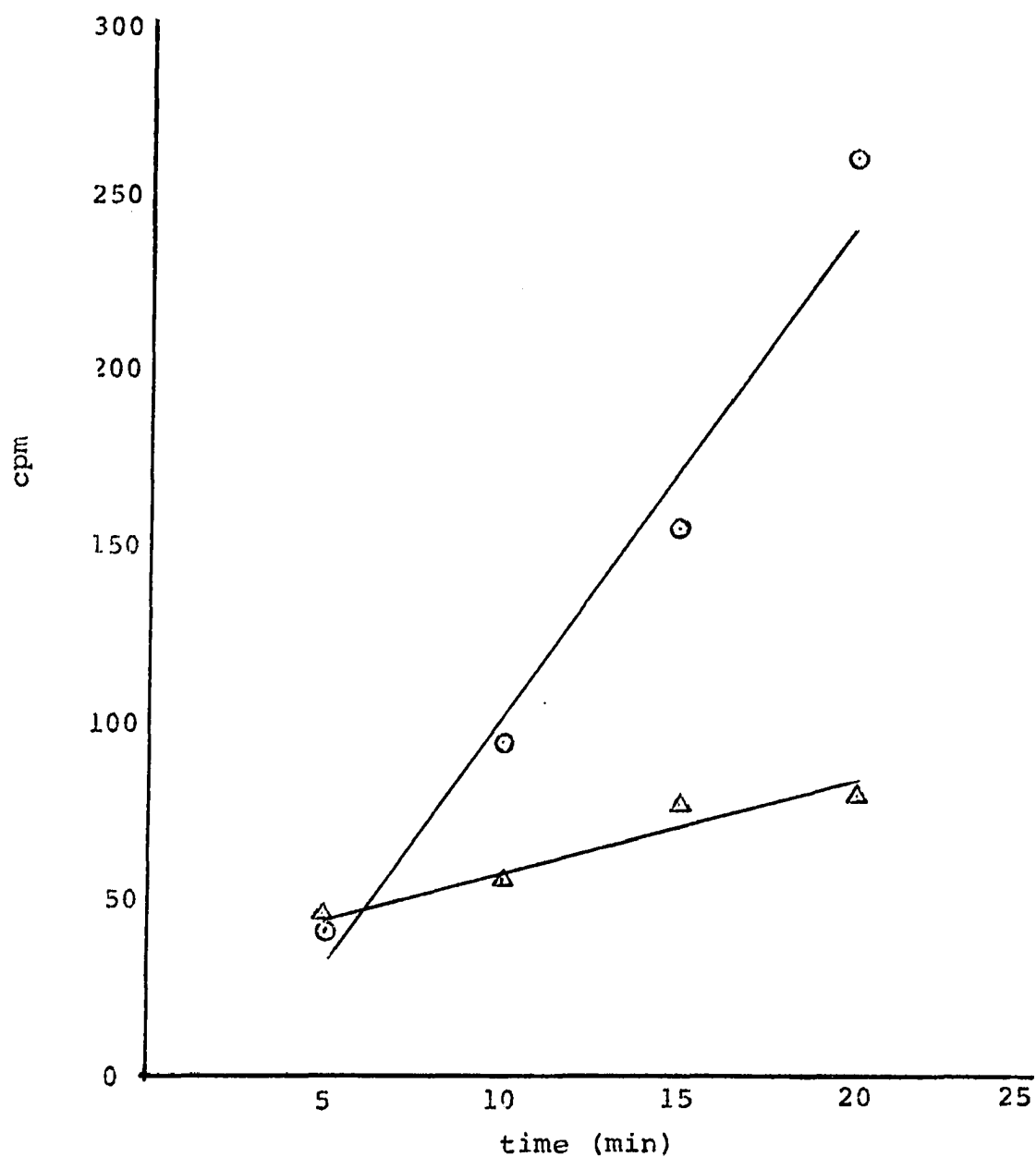


Figure 50. Assay of dextransucrase with 6,6'-dideoxy-6,6'-dichlorosucrose at ~150 mM. Preincubation for 10 minutes.

○ = control

△ = inhibitor

%I = 80

This may also be the reason that α -D-glucopyranosyl fluoride is an inhibitor at concentrations of 30 mM, i.e., it is not only acting as a substrate but also as a glucosylating agent at points other than the glucosylating site normally utilized as such in the active site of the enzyme, thereby rendering the enzyme inactive. In the case of α -D-xylopyranosyl fluoride, if the nucleophile at the active site of dextransucrase normally utilized to form the enzyme-glucosyl complex were to react with α -D-xylopyranosyl fluoride, then it might be rendered inactive towards any further reaction steps in the mechanism of the polymerization reaction. Due to the fact that the xylosyl group, unlike the glucosyl group, carries no potentially nucleophilic C-6 hydroxyl group, it is necessary to form the α -1,6-glucosidic bond of dextran, and therefore cannot undergo any further reaction. This is shown in Figure 51.

The inhibition produced by xylosyl fluoride could conceivably be reversed by prolonged incubation with substrate if the sucrose could approach the active site and/or binding site(s); however, if any nucleophilic group, (other than the active site group(s)) can be found at or near the binding site that could react with xylosyl fluoride, this then might block the entrance of any sucrose. In fact, this may be the only reaction needed to produce the inhibition seen with xylosyl fluoride.

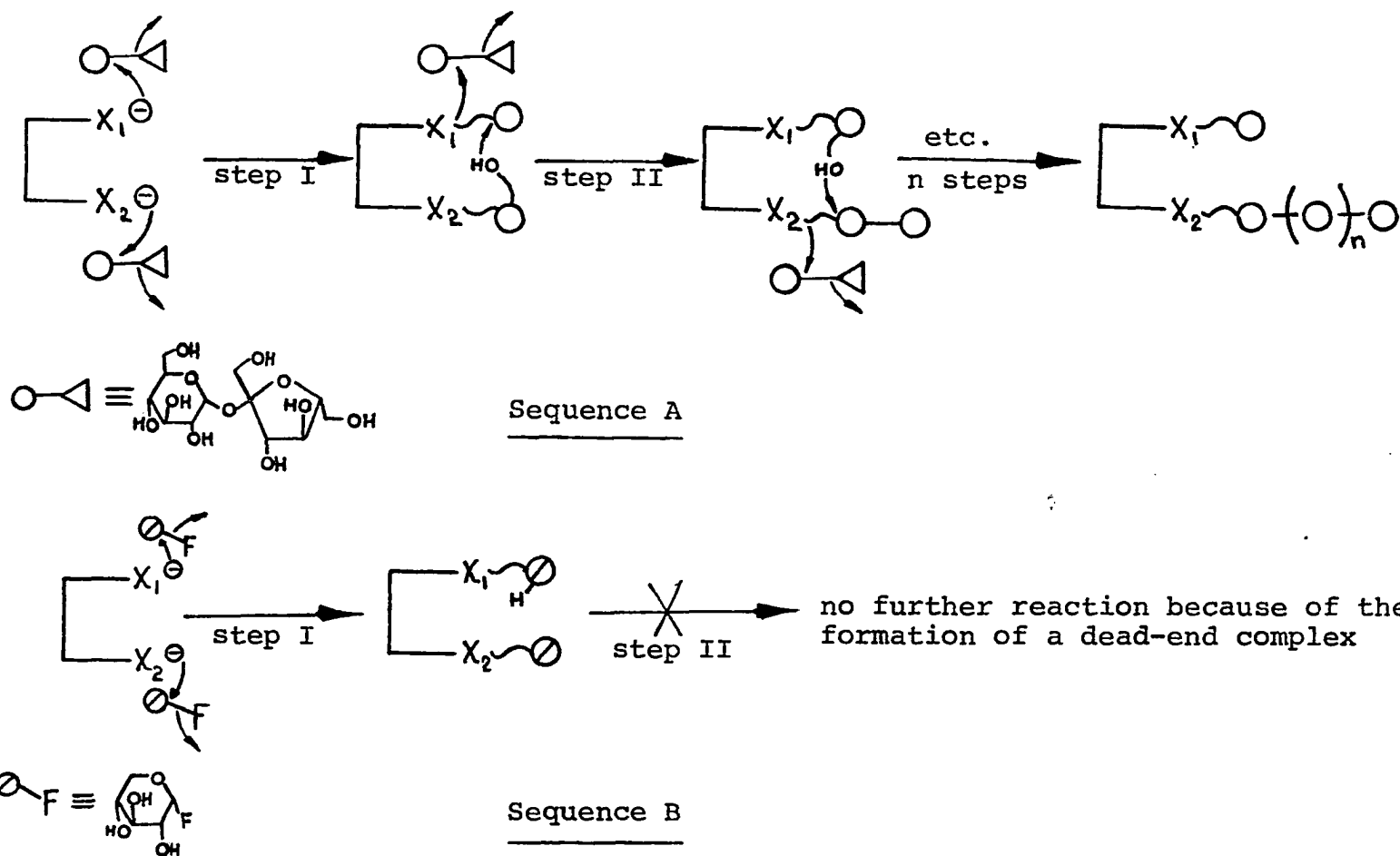


Figure 51. Sequence A - normal dextran synthesis; Sequence B - possible mechanism for inhibition by xylosyl fluoride.

The inhibition observed with 6,6'-dibromo-6,6'-dideoxysucrose could conceivably be a direct result of the fact that this substance has two very good leaving groups in its structure making it susceptible to an S_N2 type reaction at positions C-6 and C-6'; thereby, through alkylation inactivating the enzyme. This process could be occurring at the active site as illustrated in Figure 52. In order to carry out the polymerization reaction, it is necessary for the C-6 hydroxyl group to act as a nucleophile and carry out an attack on C-1 of the opposing glucosyl unit on the enzyme. This reaction would be greatly facilitated if the hydroxyl group were to become a more potent nucleophile than it ordinarily is. The most plausible method of accomplishing this is to abstract the proton via hydrogen bonding to an appropriate hydrogen bond acceptor on the enzyme, i.e.- a nucleophile such as a carboxyl, amino or an imidazole group. This would generate a much more potent alkoxide type nucleophile which would make the attack on C-1. If the hydroxyl group is not present, but a good leaving group is present, the nucleophile could possibly displace the leaving group forming in the process a covalent bond to the carbon atom, C-6, and thereby inactivate the enzyme towards any further reactions with substrate molecules. This sequence would constitute an

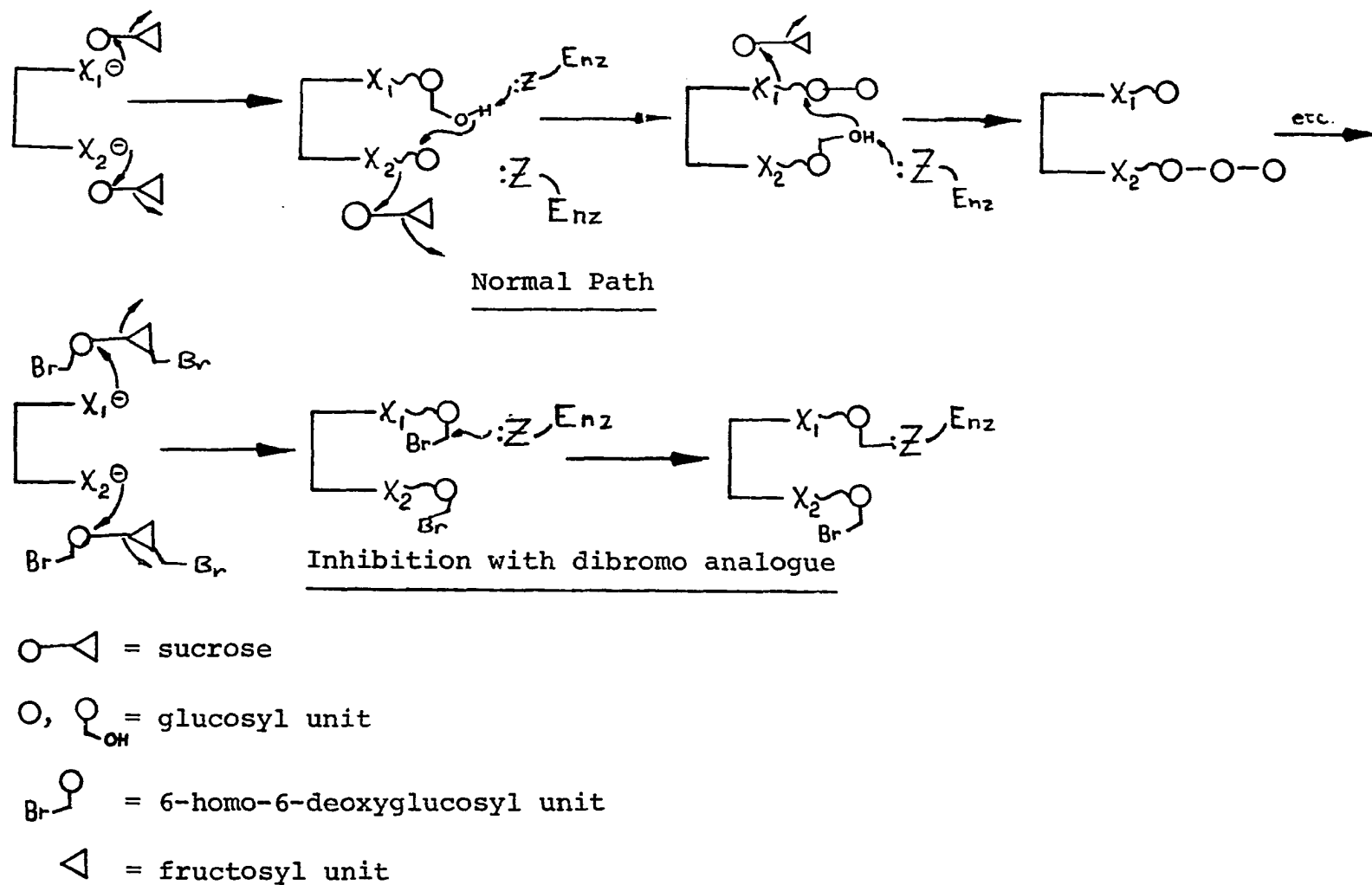


Figure 52. Proposed mechanism of inhibition of dextran sucrose transferase by alkylation of a nucleophilic group, Z, at the active site with 6, 6'-dibromo-6,6'-dideoxysucrose.

irreversible inactivation of the dextransucrase. There are, of course, probably several other points in the enzyme where alkylation could occur; however, due to the structure of the inhibitor it would be expected to have affinity for the active site and alkylation would occur most rapidly there.

In the case of the inhibitor(s) produced by the reaction of potassium fluoride on tri-0-tripsylsucrose derivatives (see Fig. 24, p. 71) a similar mechanism can be used to explain the observed results with some of the compounds present. It is highly probable that the reaction in question results in the formation of derivatives of sucrose containing at least one olefinic bond between C-5 and C-6 or between C-5' and C-6' and anhydro and fluorinated derivatives formed. If a derivative containing the C-5 to C-6 olefinic bond is formed, and this substance is bound by the enzyme, then it is likely that alkylation of the enzyme will occur with the vinyl ether acting as the alkylating agent as shown in Figure 53.

In addition to the vinyl ethers, the other substances formed in the reaction of fluoride with tri-0-tripsylsucrose derivatives, such as the anhydro derivatives, could be acting as competitive noncovalent inhibitors.

The lesser degree of inhibition observed with the 6,6'-dideoxy-6-6'-dichlorosucrose can be attributed to the fact that the chloro group is not as easily displaced as the

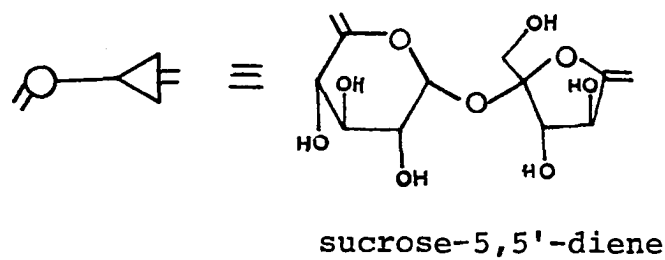
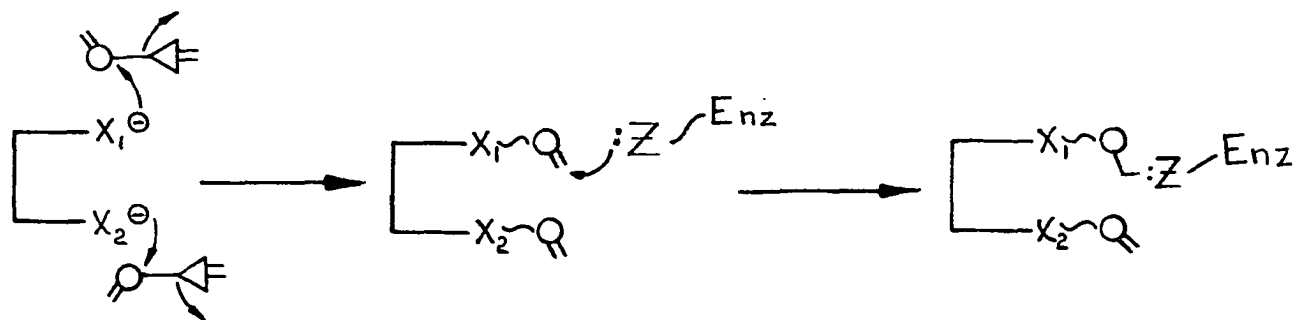


Figure 53. Possible mechanism of inhibition of dextran sucrose by compounds such as sucrose-5,5'-diene.

bromo group in S_N2 reactions, and it is therefore, not as effective an alkylating agent as the 6,6'-dibromo-6,6'-dideoxysucrose.

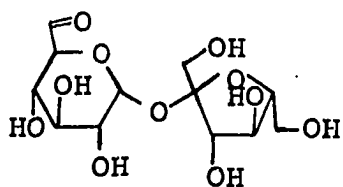
The inhibition observed with 6,6'-dideoxy-6,6'-difluorosucrose cannot be due to alkylation occurring at C-6 and/or C-6' because the fluoro group is very difficult to displace. Further, besides the formation of dead end complexes with the difluorosucrose, it is also possible that it is inhibiting the enzyme competitively by binding at the sucrose binding site. The same would be true for the 6-monodeoxy-6-monofluorosucrose.

The hypothesis presented for the reasons of the observed effects of the various sucrose analogues on dextranase lend themselves to experimental verification. In the case of α -D-xylopyranosyl fluoride the preparation described for its synthesis could be carried out using ^{14}C -labeled xylose and purifications could be attained on preparative thin layer chromatography plates. The radioactive preparation could then be incubated with dextranase and the resulting complex chromatographed on Sephadex (for example) to remove any remaining uncomplexed $[^{14}\text{C}]\text{-}\alpha\text{-D-xylopyranosyl fluoride}$. The complex could then be assayed for radioactivity and in this manner a determination could also be made of the number of moles

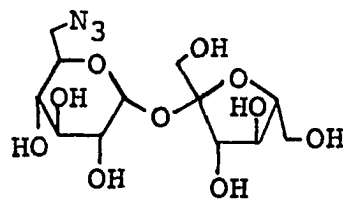
of α -D-xylopyranosyl fluoride complexed per milligram of protein. If enough enzyme could be reacted with an amount of ^{14}C - α -D-xylopyranosyl fluoride sufficient for facile detection and if the complex was covalent, as speculated, the complex could then be treated with a variety of proteases and the point or points of attachment of the ^{14}C - α -D-xylopyranosyl fluoride might be determined. A similar procedure could conceivably be carried out for each of the other sucrose analogues yielding useful information about the nature of the active site of dextranucrase. Under the most favorable conditions an identification of the true enzyme glucosyl complex might be ascertained.

In addition to the compounds used in this study, several other materials could conceivably be utilized as either alternative substrates or substrate analogue inhibitors. Some of these are illustrated in Figure 54. Compounds such as these could lead to more information concerning the requirements for the binding of sucrose onto dextranucrase and, in addition, the requirements for the reaction to proceed. The substances described in Figure 34 (page 92) could be synthesized as shown in Figure 55.

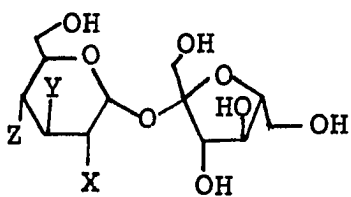
The definitive structure of 6,6'-dideoxy-6,6'-difluorosucrose was obtained by ^{13}C and ^{19}F -NMR (see Figures 19 & 20, pages 58 and 60). A comparison of the ^{13}C -NMR spectra of sucrose and 6,6'-dideoxy-6,6'-difluorosucrose shows that



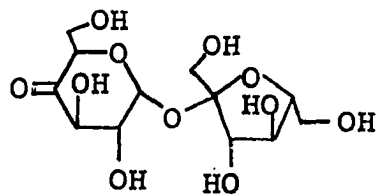
(I)



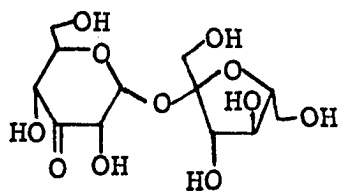
(II)



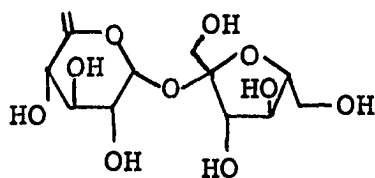
(III)



(IV)



(V)



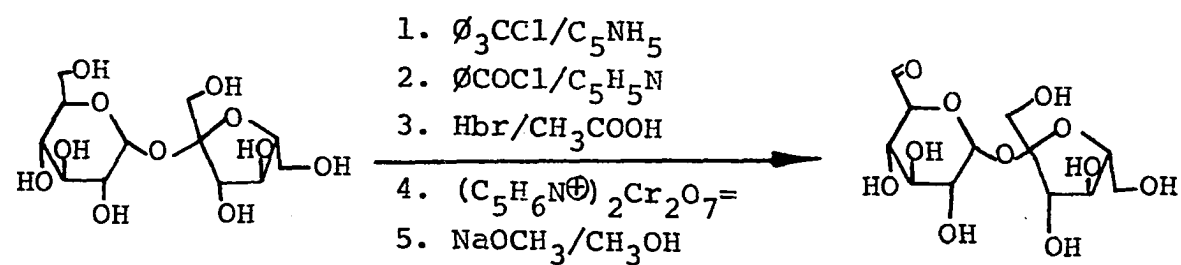
(VI)

X = (H, OH, F)

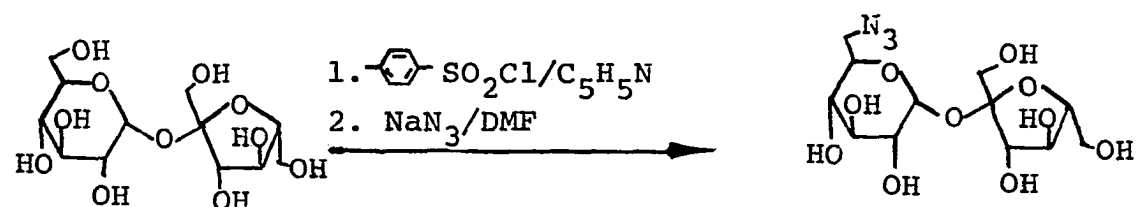
Y = (H, OH, F)

Z = (H, OH, F)

Figure 54. Possible inhibitors of dextransucrase.



6-aldosucrose



6-deoxy-6-azidosucrose

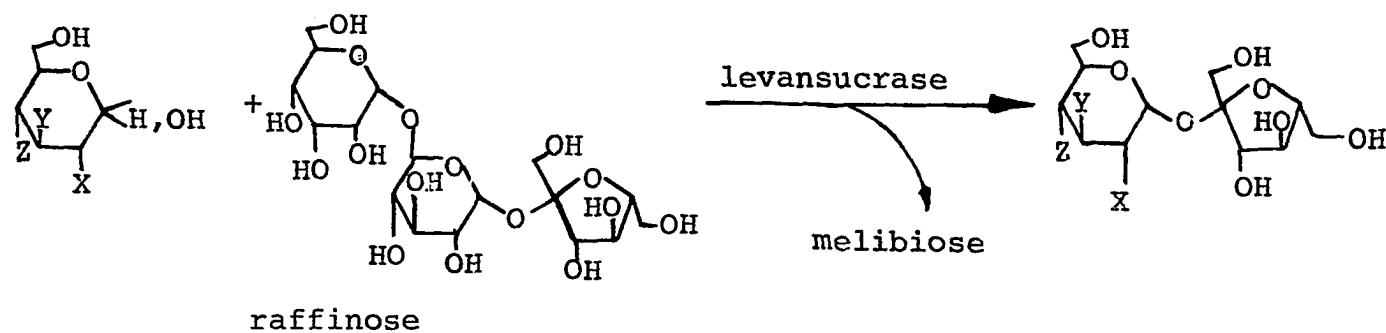


Figure 55. Proposed synthetic routes for the synthesis of the various analogues.

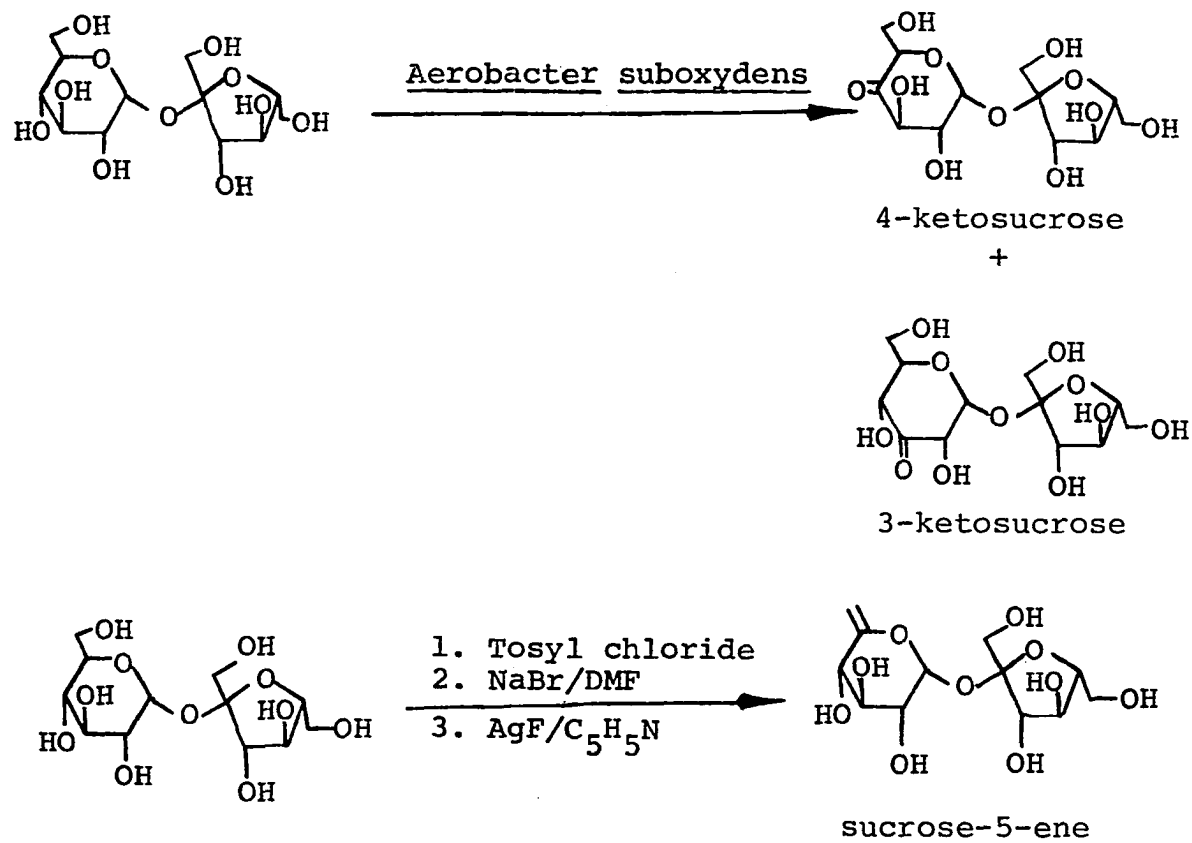


Figure 55. Continued.

the new compound has a sucrose-like structure that was substituted at C-6 and C-6' in that the compound has peaks at 105.57 ppm, 93.98 ppm, and 62.88 ppm corresponding to sucrose peaks at 104.87 ppm, 93.33 ppm, and 62.67 ppm which arise from carbons 2', 1, and 1' respectively. Conspicuously absent from the spectrum of the synthesized compound are peaks at 63.53 ppm and 61.37 ppm, which correspond to carbons 6 and 6' of sucrose. Further assignments of the peaks in the ^{13}C -NMR spectrum was complicated due to a variety of carbon-fluorine couplings. ^{19}F -NMR, however, provided confirmatory evidence for the structure.

The ^{19}F -NMR spectrum (see Figure 27, page 80) shows two distinct sextets centered at 223.633 ppm and 223.958 ppm. These two sextets are due to signals arising from a fluorine atom at C-6 coupled to the two hydrogens at C-6 resulting in a triplet which is further split by the lone hydrogen on C-5 leading to a sextet; the same thing is true for the fluorine at C-6', split into a triplet by the two hydrogens on C-6' and further split by the lone hydrogen on C-5'. Substitution at no other carbons would give rise to these sextets. For example, if substitution had occurred at C-1' a unique triplet would have resulted and substitution for any of the secondary hydroxyls would have given rise to a higher order multiplet with a different pattern for the intensities. The

synthesized compound, therefore, is sucrose substituted at C-6 and C-6' by fluorine.

The fluorination reaction with DAST might have been expected to result in the substitution of all three of the primary hydroxyl groups for fluorine to yield 6,1',6'-trideoxy-6,1',6'-trifluorosucrose. Under our conditions, 6,6'-dideoxy-6,6'-difluorosucrose resulted as the major product and follows the pattern of substitution on sucrose elucidated by Hough and coworkers (15-18) in which it was found that C-1' was the last and most difficult to be substituted of the three primary hydroxy bearing carbons. With slight changes in solvent, temperature and reaction time, 6,1',6'-trideoxy-6,1',6'-trifluorosucrose, however, should result from the synthesis.

The substitution of hydroxyl groups by fluorine is usually a difficult halogenation procedure due to the low nucleophilic yet basic character of the fluoride ion, which usually gives rise to elimination reactions and other reactions such as deacylation followed by O-alkylation. These reactions require the use of nonbase labile protecting groups, such as benzyl ethers, rather than acetates or benzoates which can be more easily removed. The use of DAST, with a properly substituted sucrose intermediate, has provided us with a means around these problems to give

6,6'-dideoxy-6,6'-difluorosucrose which is of interest in studying biological and enzymatic reactions involving sucrose.

In the study of the mechanism of action of dextran-sucrase and other similar enzymes, there is one inherent difficulty in obtaining some types of information otherwise obtainable from enzymes such as those utilizing pyridoxal phosphate, namely the lack of any type of easily distinguishable chromophore which can be used as a probe into the mechanisms occurring at the active site. It is possible now, however, to overcome this difficulty in some respects and to provide information about the enzyme without the use of substrate analogues, which might introduce variations that are not part of the normal sequence of events at the active site. The method to overcome these problems could be the use of ^{13}C Fourier Transform NMR with various cryo-enzymological techniques. An example of this is as follows: ^{13}C labeled sucrose obtained either by chemical means, e.g., complete synthesis, biological means, or more likely a combination of the two, e.g., coupling of ^{13}C -labeled glucose to fructose using levansucrase, could be mixed with dextransucrase at -70°C , probably in DMSO/buffer 8/1 v/v or other appropriate mixtures determined experimentally, and a ^{13}C -NMR taken of this mixture at this temperature. The

temperature could then be raised slowly and a ^{13}C -NMR spectrum obtained at intervals or continuously if possible until the signals observed changed. This change would probably first be noticed in the signal resulting from the C-1 of the glucosyl unit as the bond to the fructosyl unit was broken and the bond to the enzyme formed. The temperature could then be lowered, preventing any further reaction so that the spectrum could be run several times to allow for a good spectrum to be obtained by Fourier Transform NMR techniques. Since the sucrose is more heavily labeled than the 1.1% natural abundance of ^{13}C found in sucrose, the spectrum could be obtained in a much shorter period of time. If the bond to the glucosyl unit and the enzyme is a phosphate linkage, this then could be ascertained by knowing the absorbance or shift of C-1 of glucose-1-phosphate in both the α and β configurations.

Similar experiments could be carried out using ^2H NMR. In addition, various fluorinated analogs could be synthesized and similar techniques could be used if the appropriate ^{19}F -NMR spectra were first obtained as controls.

IV. CONCLUSIONS

1. Several analogues of sucrose were synthesized for the purpose of studying the mechanism of action of dextran-sucrase.
2. Among these analogues were three novel compounds namely 6,6'-dideoxy-6,6'-difluorosucrose, 6-deoxy-6-fluorosucrose, and 6'-deoxy-6'-fluorosucrose. The structures were confirmed by ^{13}C -NMR, ^{19}F -NMR and various chromatographic techniques.
3. All of the compounds inhibited the action of dextran-sucrase to some degree, some being more potent inhibitors than others.
4. The most potent inhibitors appeared to be those analogues of sucrose which could effectively act as alkylating agents for dextran-sucrase, such as 6,6'-dideoxy-6,6'-dibromosucrose.
5. It is postulated that the 6,6'-dideoxy-6,6'-difluorosucrose inhibits dextran-sucrase by forming a covalent dead-end complex.
6. This has led to the possibility of further probing the enzyme by use of these and other similar materials.

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